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Original article

Perindopril sensitizes hepatocellular carcinoma to chemotherapy: A possible role of leptin / Wnt/ β-catenin axis with subsequent inhibition of liver cancer stem cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death. The major challenge in managing HCC is the resistance to chemotherapy. Leptin hormone is associated with different oncogenic pathways implicated in drug resistance. Angiotensin II was found to decrease the production and secretion of leptin.

Objective: This study investigated the potential role of an ACEI perindopril as a chemosensitizer agent to sorafenib.

Method: HCC was induced in mice using a single dose of diethylnitrosamine DENA (200 mg/kg) followed by phenobarbital 0.05% in drinking water for 16 weeks. Mice were then treated with perindopril (1 mg/kg/day), Sorafenib (30 mg/kg/day), or both of them for another four weeks. Leptin, VEGF, MMP-9, Cyclin D1, EpCAM, and β-catenin were measured using immunoassay while Wnt and ALDH1 were assayed using western blotting assay.

Results: Perindopril whether alone or in combination with sorafenib decrease liver enzymes and preserve the liver architecture. Our study revealed that perindopril significantly increased the antineoplastic, antiangiogenic as well as anti-metastatic effects of sorafenib. This effect was correlated with the down-regulation of the leptin / Wnt / β-catenin pathway and overexpression of ALDH1 while downregulation of EpCAM

Conclusion: This study presents perindopril as a potential chemosensitizer agent that works through decreased expression of the leptin / Wnt / β-catenin pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most dangerous cancers. It is the third leading cause of cancer-related mortality worldwide (Forner, 2012). The current therapeutic protocols include surgery for localized tumors or liver transplantation (Carr, (2004).) Other medical procedures such as radiofrequency, percutaneous ethanol, acetic acid injection, or interstitial laser coagulation are effective only in the early stages of tumors (Carr, (2004).) Late stages of HCC are treated with systemic chemotherapy and some mTOR inhibitors such as sorafenib. The latter could promote apoptosis, inhibit angiogenesis, and suppress tumor proliferation (Tang et al., 2020).

The major molecular mechanism of sorafenib induced suppression of tumor proliferation is the downregulation of Ras/Raf/MEK/ERK signaling pathways. Moreover, its antiangiogenic activity is

Abbreviations: **ABC**, ATP binding box; **ACEI**, Angiotensin converting enzyme inhibitors; **ALT**, Alanineaminotransferase; **APC**, adenomatous polyposis coli protein; **AST**, Aspartate aminotransferase; **ALDH1**, Aldehyde dehydrogenases 1; **ANG II**, Angiotensin II; **DENA**, Diethylnitrosamine; **EpCAM**, Epithelial cell adhesion molecule; **GSK 3β**, glycogen synthase kinase-3β; **HCC**, Hepatocellular carcinoma; **LCSC**, Liver cancer stem cells; **MMP-9**, Metalloproteinase-9; **mTOR**, Mammalian target of rapamycin; **PBS**, Phosphate-buffered saline; **PDGFR-β**, platelet-derived growth factor receptor; **PRIN**, Perindopril; **SOR**, Sorafenib; **VEGF**, Vascular endothelial growth factor.

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related to the targeting of platelet-derived growth factor receptor (PDGFR-β) and vascular endothelial growth factor receptor (VEGFR)(Wilhelm et al., 2004). Although these potential molecular targets are affected by sorafenib, poor response and high incidence of relapse are still of significant importance (Witjes, Verhoef, Verheul, & Eskens, 2009). This is maybe due to different resistance mechanisms such as ATP binding box (ABC) transporters which facilitate drug efflux out of cancer cells (Beretta, Cassinelli, Pennati, Zuco, & Gatti, 2017), activation of autophagy, and tumor escape from the immune response (Luo et al., 2016); (Dong et al., 2019).

The current challenge in HCC management is to control chemotherapy resistance, recurrence, and metastasis. Recently, it has been proposed that cancer may originate from normal stem cells differentiation. Liver cancer stem cells (LCSC) are found in tumor tissues as a rare group of cells that has a high potential for endless multiplication (Ma et al., 2008). It has been reported that LCSC is responsible for chemotherapy resistance and tumor relapse (Feng et al., 2020). These types of cells had been identified by different markers such as CD 133 and epithelial cell adhesion molecule (EpCAM) (Ma et al., 2008; Terris, Cavard, & Perret, 2010). This type of cells utilizes a certain signaling pathway that is essential for its renewal capacity such as Wnt, Notch, and Hedgehog signaling pathways (M. Wang et al., 2009);(Cheng et al., 2009). Dysregulation of these molecular pathways may be an important target to inhibit LCSC activity, decrease resistance to chemotherapy and retard the recurrence of HCC.

Leptin is one of the regulating hormones that is associated with chemotherapy resistance and induction of cancer stem cells. It has been reported that leptin is secreted at a higher level from hepatoma tissues and hepatoma cell lines compared to normal liver tissue or hepatocytes (Wei et al., 2016). The suggested mechanism of leptin oncogenic effect is thought to be done through upregulating of Wnt/β-catenin as well as Notch signaling pathway (Harbuzariu et al., 2017; Yan, Avtanski, Saxena, & Sharma, 2012). These signals are particularly important for the proliferation of LCSC.

Patients with HCC have an elevated level of angiotensin II. Angiotensin II (ANG II) is known to promote leptin production and secretion. Recently, angiotensin converting enzyme inhibitors (ACEI) in particular perindopril has been found to decrease leptin plasma level (Mathai, Naik, Sinclair, Weisinger, & Weisinger, 2008). Moreover, it has been reported that perindopril induced leptin suppression is more potent compared to enalapril (Werida, Khairat, Khedr, & El-Sisi, 2020).

This relation raised a question about the role of leptin in the anticancer effect of ACEI and possible crosstalk between inhibition of ANG-II and consequently, leptin in decreasing drug resistance and LCSC activity.

We hypothesize that inhibition of angiotensin II by ACEI may decrease the activity of LCSC through inhibition of leptin oncogenic potential thus sensitizing HCC to chemotherapy such as sorafenib. To validate this hypothesis, we investigate the antineoplastic and chemosensitizing effect (if any) of perindopril as one of the ACEI in experimentally induced HCC in mice.

2. Method

2.1. Drugs and chemicals

Diethylnitrosamine (DENA) and Phenobarbital were purchased from Sigma- Aldrich (St. Louis, MO, USA). Sorafenib (Sor) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Perindopril arginine was a kind gift from servier Egypt Company (Cairo, Egypt). The chemicals were prepared as follows:

0.1 ml of diethylnitrosamine concentrated solution (1gm in 0.5 ml) was dissolved in 25 ml saline to produce a working solution (8 mg/ 1 ml) whereas 5 mg Perindopril arginine was dissolved in 20 ml saline to produce a working solution (1 mg / 4 ml) and Sorafenib tosylate was suspended in 0.2% CMC to produce a homogeneous suspension according to the method of Sharma, *et al.* (Sharma et al., 2020).

All other chemicals used herein were obtained from the El-Gomhouria company for trading chemicals and medical appliances (Cairo) and were of the highest quality/analytical grade.

2.2. Animals

Sixty Male mice weighing 20–30 g were obtained from the National Research Center (Dokki, Giza, Egypt) and housed in a pathogen-free animal house in plastic cages with rice husk bedding. Mice were kept in the temperature range of 25 [±2] °C, with a ≈ 50% relative humidity, and a 12-hr light: dark cycle. All mice had *ad libitum* access to rodent chow and water. The Ethics Committee of Kaferelsheikh University has approved the study protocol including the use of animals during the experiment.

The experimental procedures were done in compliance with the ARRIVE guidelines in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments**.**

2.3. Experimental protocol

Sixty male mice were used in this study. Forty mice were injected with a single dose of DENA (200 mg/kg) intraperitoneally. One week later phenobarbital was added to drinking water at a concentration of 0.05% for 16 weeks(Shahjahan, Vani, & Shyamaladevi, 2005). The mice were then allocated into four groups. The first group of mice (DENA + Sor) was treated with sorafenib 30 mg/kg/day orally (Kuczynski, Lee, Man, Chen, & Kerbel, 2015). The second group of mice (DENA + PRIN) was gavaged with perindopril (1 mg/kg/day) (Saber et al., 2018) orally while the third group of mice (DENA + SOR + PRIN) was given both drugs with the same mentioned doses. The fourth group of mice (DENA) was left without further treatment. Twenty mice were served as control groups. Ten mice of them were gavaged with sorafenib 30 mg/kg/-day and perindopril (1 mg/kg/day) orally (SOR + PRIN) while the other 10 mice (control group) were treated with vehicles **(Fig-1).**

The used HCC model in our study was designed as a two-stage model'' initiation and promotion'' where the genotoxic compound DENA is used as the initiator and phenobarbital is used as the promoter. Phenobarbital is an enzyme inducer that increases the cytochrome expression P450 by about 100-fold, this results in an enhanced effect of DENA and induction of oxidative stress (Waxman & Azaroff, 1992); (Dostalek et al., 2008) (See Fig. 1).

2.4. Preparation of blood and tissue homogenate

At 24 h after the last dose of drug/vehicle injection all mice were anesthetized using diethyl ether. Blood samples were immediately obtained via cardiac puncture and collected into tubes then allowed to be clotted at room temperature for 60 min. The samples were then centrifuged (3000xg, 10 min, 4 °C), and the resultant serum in each supernatant was recovered and stored at −20 °C until analysis**.**

Mice were killed by cervical dislocation. The liver of each animal was then carefully removed and washed twice with saline. Two 0.5-cm sections of the second-largest lobe were incubated in 10% formol saline and further prepared for histopathological examination. The remaining portions of each liver were divided into four parts and individually stored at −80 °C until analyzed. Liver

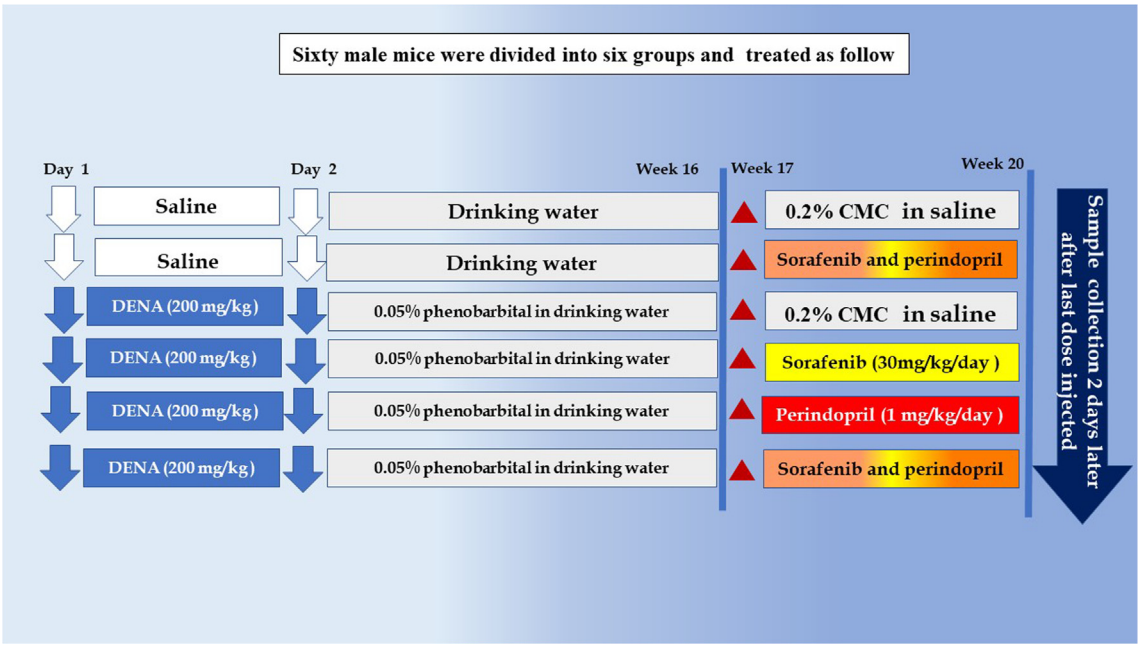

Fig. 1. Experimental design. Sixty male mice were divided into six groups and treated with (DENA, DENA + Perindopril, DENA + sorafenib, DENA + Perindopril + sorafenib) as explained in the figure. More details in the text.

homogenates were prepared in ten volumes (i.e. 0.1 g tissue in 1 ml) of cold phosphate-buffered saline (PBS, pH 7.4). Each resulting mixture was centrifuged (6000 g, 20 min, 4C○). Aliquots of the derived supernatant were used for biochemical analysis and measuring protein contents using a standard kit (Wokea Medical Supplies, Changchun, China).

2.5. Assay of liver enzymes

The assays of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were done using Biodiagnostics kits (Biodiagnostics, Dokki, Giza, Egypt) that measure the amount of pyruvate or oxaloacetate produced from 2,4-dinitrophenylhydrazine respectively.

2.6. Immunoassay of Leptin, VEGF, MMP-9, cyclin D1, β-catenin, and EpCAM

Serum leptin was assayed quantitatively using enzyme-linked immunosorbent assay (ELISA) technique according to kit instructions (ab100718 Leptin Mouse ELISA Kit, Abcam, UK). The minimum detectable limit was 4 pg/mL.

Proliferation, angiogenesis, and metastasis are considered the hallmarks of cancer. This study assessed cyclin D1 as a marker of cell cycle progression and proliferation. It mainly controls the G1/S-phase transition. It also allows cells to accelerate through the cell cycle and consequently promotes cell proliferation (Zhang, Wang, & Li, 2016). Vascular endothelial growth factor (VEGF) and metalloproteinase-9 (MMP-9) were selected as markers for angiogenesis and metastasis. The expression levels of the proteins were assayed according to ELISA kits instruction (ELISA genie, Dublin, Ireland SKU: MOFI00255), (MyBioSource, San Diego, USA, Catalog Number: MBS825096), and (CUSABIO, Wuhan, Hubei China. Catalog Number: CSB-08007 m) respectively. The detection range of cyclin D1 was 0.156–10 ng/ml with a sensitivity level of about 0.094 ng/ml. The sensitivity level for VEGF was less than 7 pg/ml while the MMP-9 sensitivity level was less than 0.552 ng/ml.

The expression of the β-catenin and EpCAM protein in liver tissues was done also using ELISA method according to the kit manufacturing instructions (CUSABIO, Wuhan, Hubei China. Catalog Number: CSB-E11307m) and (Abcam, Cambridge, USA. Catalog Number: ab242247) respectively. The sensitivity level for mouse β-catenin is typically less than 0.039 ng/ml while the detection range was 0.156 ng/ml-10 ng/ml. The detection range for the mouse EpCAM ELISA kit was 125 pg/ml – 8000 pg/ml with a sensitivity level of 6 pg/ml.

2.7. Western blot analysis

The expression of Wnt proteins and ALDH1 were done using the western blot technique as follows: Frozen liver tissue samples were prepared in a solution containing RIPA buffer (Sigma, St Louis, MO, USA) to produce a 10% w/v tissue homogenate. The resultant homogenates were then centrifuged at 14,000 rpm for 20 min at 4 °C. Protein quantification was performed with Biuret Method using biodiagnostic kits (Biodiagnostics, Dokki, Giza, Egypt).

The protein extract (40 μg/lane) was separated on a 12% SDS-PAGE gel and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed once with TBST and probed with antibodies specific to Wnt (dilution, 1:1,000; cat no. sc-179755), ALDH (dilution, 1:1,000; cat no sc-166362) β-actin (dilution, 1:1,000; cat no. sc-130656) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) Followed by incubation with an HRP-conjugated secondary antibody (Zhongshan Bio, Beijing, China). The protein bands were analyzed using image J-win 32 (version 1.48u; MD, USA).

2.8. Histopathological examination of liver tissues

Liver samples were removed from the liver of each mouse and fixed for twenty-four hours in 10% formol saline. Tissues were washed using tap water, then serial dilutions of alcohols (methyl, ethyl, and absolute) were used for dehydration.

Specimens were cleared in xylene and embedded in paraffin at 56 °c in a hot air oven for twenty-four hours. Sections of paraffin beeswax blocks at 4 μm thickness were prepared by a microtome. The obtained tissue sections were collected on glass slides, deparaffinized then stained with hematoxylin & eosin stain for examination under the light electric microscope(Banchroft, 1996).

2.9. Statistics

All data are displayed as means ± SD. Statistical significance between results from all groups was done using one-way analysis of variance (ANOVA) with Tukey's test as a post-hoc test. A p-value $\leq$ 0.05 was considered significant. All analyses were performed using Prism software (v.6.0, Graphpad, San Diego, CA).

3. Results

3.1. Perindopril/sorafenib combination has a hepatoprotective effect

DENA treated mice showed significant *($P \leq 0.001$)* increases in ALT, AST serum levels (111.4 ± 12.99, 267.9 ± 35.43) compared to control mice (38.60 ± 5.441, 40.50 ± 8.91). Sorafenib, Perindopril, and combination of the two drugs displayed a significant ($P \leq 0.001$) decrease compared to DENA treated mice (91.90 ± 5.49, 74 ± 4.9, 68.7 ± 6.9) for ALT and (204.7 ± 1.97, 79.9 ± 6.74, 99.11 ± 6.4) for AST respectively. Mice treated with drugs combination exhibited a potent hepatoprotective effect compared to each drug alone ($P \leq 0.001$). The mice treated with sorafenib and Perindopril without DENA showed significant ($P \leq 0.001$) increased levels of both ALT and AST serum levels (70.50 ± 11.60, 86.30 ± 4.86) compared to control ones **(Fig-2 a and b)**.

3.2. Perindopril potentiates the antiangiogenic and antimetastatic effect of sorafenib

To detect the effect of our drugs on cancer angiogenesis and metastasis we assessed the expression of VEGF and MMP-9 as biomarkers for those characteristic events in hepatocellular carcinoma. The mice treated with DENA showed significant ($P \leq 0.001$) inductions of both VEGF and MMP-9 (112 ± 12.56, 23.86 ± 2.63) respectively compared to control mice (27.24 ± 4.75, 8.25 ± 1.011). The present study showed that Sorafenib or perindopril exerted antiangiogenic (61 ± 5.61, 62.21 ± 6.22) and antimetastatic (14.22 ± 1.34, 13.92 ± 1.809) effects compared to DENA however the effect of each drug was nearly similar. On the other hand, when both drugs were used as a combination therapy the effects were significantly (49.46 ± 5.35, 10.24 ± 1.535) ($P \leq 0.05$) superior compared to each drug alone (Fig. 3 **A and B**).

3.3. Impact of sorafenib /perindopril combination on cell cycle

The expression of Cyclin D1 is a crucial factor for liver cells growth and initiation of HCC as well. This factor could promote HCC by directing cell cycle to enhanced proliferation (Che et al., 2012). The expression of cyclin D1 in DENA treated mice showed about 4.5 fold elevation (9.41 ± 0.8) compared to normal mice (1.7 ± 0.22). Therapeutic interventions using Sorafenib, perindopril or both of them resulted in 53%, 55%, and 68 % inhibitions in cyclin D1 expression in liver tissues respectively compared to HCC mice ($P \leq 0.001$).

It should be noted that sorafenib and perindopril exerted a similar effect on cyclin D1 however the combination of the two drugs improves their activity compared to each drug alone ($P \leq 0.001$) **(**Fig. 3**C)**.

3.4. Perindopril dampened leptin serum level

Mice treated with DENA and those treated with sorafenib showed a significant *($P \leq 0.001$)* increase in serum leptin level (21.05 ± 3.79, 19.01 ± 3.25) compared to control mice (5.83 ± 1.25). On the other hand, perindopril whether alone or combined with sorafenib showed a significant ($P \leq 0.001$) decrease in leptin serum level (5.61 ± 2.61, 6.05 ± 2.84) compared to DENA treated mice. Mice treated with combination therapy showed a non-significant change compared to the perindopril group. There was a non-significant difference between perindopril treated mice and control ones **(**Fig. 4**A).**

3.5. Effect of sorafenib /perindopril combination on the Wnt/β-catenin pathway

In this study, we assess the possible effect of our therapeutic combination on the expression of the Wnt / β-catenin pathway as one of the signaling pathways that have a pivotal role in the activation and proliferation of stem cells in the liver (W. Wang, Smits, Hao, & He, 2019; Zhang et al., 2016) Our results provide evidences that the Wnt/β-catenin pathway is highly expressed in DENA treated mice compared to control mice. The expression of Wnt in those mice was nearly 3.43 fold higher compared to control ones. Consequently, the tissue level of β-catenin protein (9.22 ± 1.07) was significantly **(**$P \leq 0.001$) high when compared to control mice (3.72 ± 0.86). The dual therapy with sorafenib and perindopril showed potent inhibition of this signaling pathway that is expressed as 50.02 % inhibition of Wnt expression and 54.86 % decrease in β-catenin level compared to DENA.

We should confirm that this effect was significantly ($P \leq 0.05$) more potent when compared to the activity of each drug alone. The mice treated with sorafenib and perindopril conserved normal level of β-catenin in liver tissues (Fig. 4 B & C).

3.6. Effect of perindopril and or sorafenib on LCSCs marker

Epithelial cell adhesion molecule (EpCAM) positive HCC cells have the feature of hepatic stem cells. These cells play a significant role in the growth and invasiveness of the tumor. Patients with EpCAM + ve cells have a bad prognosis (Yamashita et al., 2009). The current study showed that DENA treated mice induced potent expression of EpCAM reaching about 3-fold increase (598 ± 68.9) compared to control ones (153.4 ± 29). Perindopril whether used alone or incombination with sorafenib resulted in significant ($P \leq 0.001$) Downregulation of EpCAM expression by (24%, 26 %) respectively compared to the DENA group. The standard HCC therapy, sorafenib exhibited a slight decrease in EpCAM expression (554.7 ± 49.78) however the effect did not reach a significant level. We should note that there was a nonsignificant difference between mice treated with Perindopril compared to those treated with a combination therapy of Perindopril and sorafenib (Fig. 5**A)**.

3.7. Aldehyde dehydrogenase 1 expression

Aldehyde dehydrogenase is a group of enzymes that have a critical role in detoxification. One of the important isoenzymes in this group is ALDH 1 which is correlated with a potential role in stem cells and cancer stem cells. Our results pointed out that relative expression of ALDH1 is inhibited significantly with the induction of HCC using DENA while interventions with sorafenib or perindopril or both of them regained the expression of the enzyme significantly. ALDH1 expression in mice treated with the combination therapy showed a significant ($P \leq 0.001$) overexpression compared to DENA, sorafenib and perindopril treated groups **(**Fig. 5**B).**

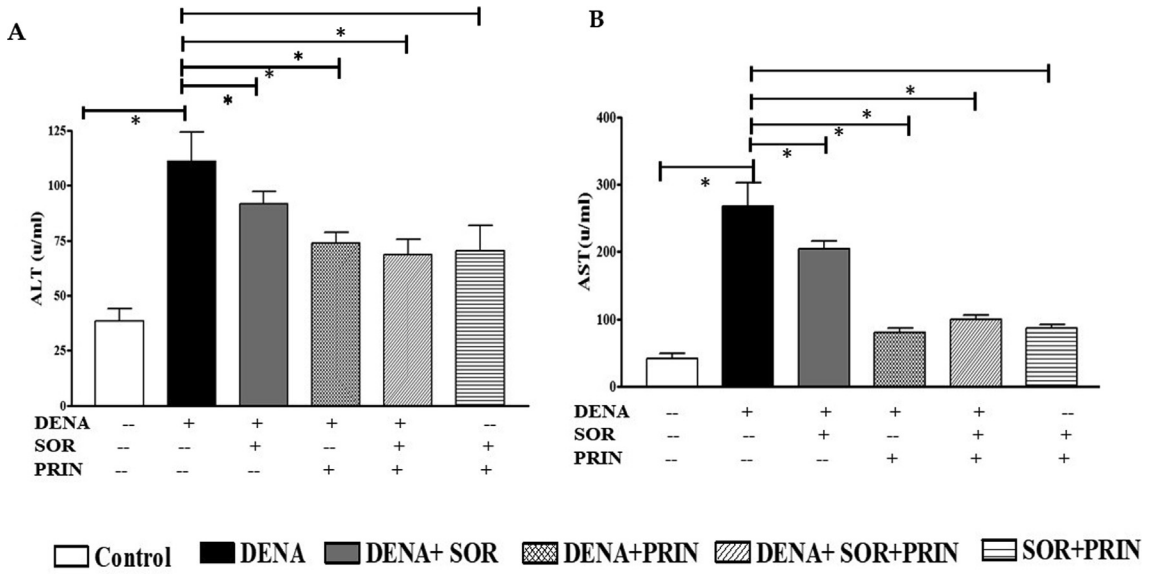

Fig. 2. Hepatoprotective effect of sorafenib/ Perindopril regimen: effect of drugs on ALT (Fig. 2 A) and AST (Fig. 2 B). Data are expressed as means ± SD. * Value significantly *($p \leq 0.001$)* different between groups. n = 10.

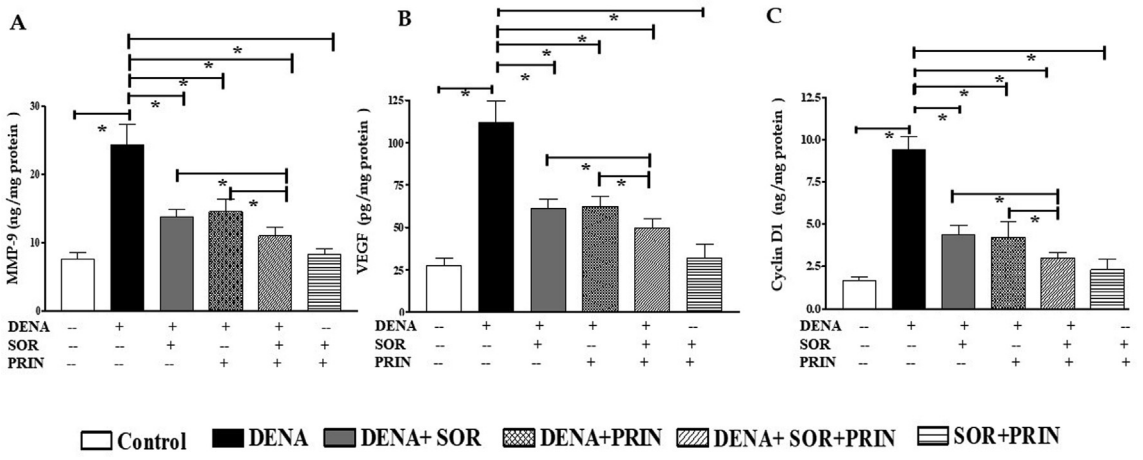

Fig. 3. Impact of sorafenib/ Perindopril regimen on metastasis, angiogenesis, and cell cycle progression. Fig. 3 illustrates the effect of sorafenib, Perindopril, and combination of them on the metastatic marker (MMP-9) (Fig. 3 A), angiogenic marker (VEGF) (Fig. 3B), and marker of cell cycle progression (cyclin D1) (Fig. 3 C) Data are expressed as means ± SD. * Value significantly *($p \leq 0.001$)* different between groups. n = 10.

3.8. Histopathological examination of liver tissues

Livers from mice in each group were first examined macroscopically. The liver from control mice appeared in normal shape with smooth margins and red color **(**Fig. 6 **A)** while livers from DENA treated mice showed multiple nodules of HCC (black arrows) **(**Fig. 6**B&C)**. Perindopril and/or sorafenib treated mice showed a soft margin with pale reddish-brown color **(**Fig. 6 **D, E&F)**.

Control mice showed normal liver architecture, as well as sections from mice treated with sorafenib and perindopril, showed preserved hepatic architecture **(**Fig. 7**A-B).** On the other hand, Liver sections from DENA treated mice displayed multiple nodules in liver tissue. Each nodule showed pleomorphic hepatocytes arranged in trabeculae (more than two cells thick. Focal spindling could be seen. More than one focal area of small cell dysplasia was also detected **(**Fig. 7**C-D).** The effect of sorafenib on liver tissues is represented in **(**Fig. 7**E)** where liver sections showed macrovesicular steatosis (mild steatosis) with a focal small area of hepatocyte degeneration. Fig. 7**F** is a presentative liver section from perindopril treated mice where normal architecture is still preserved with moderate infiltration by lymphocytes in some portal tracts and nuclear dysplasia in the form of hyperchromatic nuclei and pleomorphism could be seen in some hepatocytes. Liver sections from mice treated with combination therapy sorafenib and perindopril showed preserved architecture with some lymphocytes infiltration in portal tract and interface hepatitis but no dysplastic changes were seen **(**Fig. 7**G-H)**.

4. Discussion

One of the major challenges in HCC is the chemoresistance to chemotherapeutic agents such as sorafenib especially in the late stages (Carr, (2004).) This study examined the potential role of perindopril as one of the ACE inhibitors in decreasing HCC resistance to standard therapy (sorafenib) through its ability to modulate leptin and its consequent oncogenic effects.

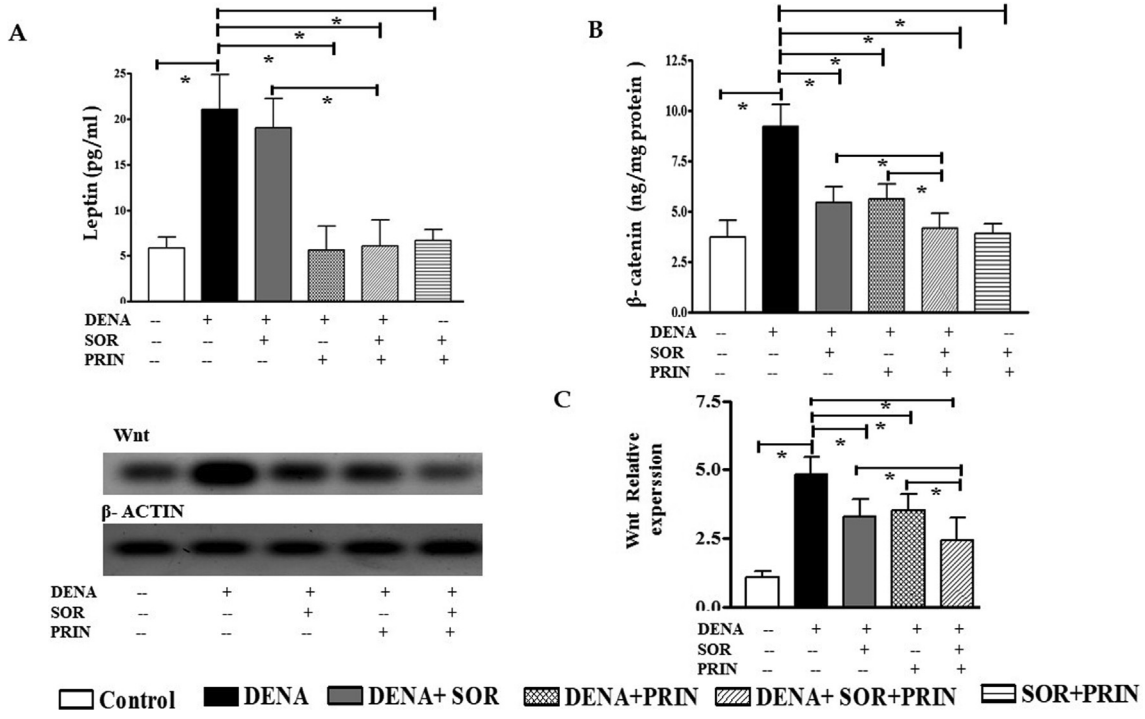

Fig. 4. leptin / Wnt / β-catenin axis as a molecular target of sorafenib/ Perindopril regimen in DENA induced HCC. Fig. 4 illustrates the effect of sorafenib, Perindopril, and combination of them on Leptin (Fig. 4A), β catenin (Fig. 4B) and wnt expression (Fig. 4 C). Data are expressed as means ± SD. * Value significantly *($p \leq 0.001$)* different between groups. n = 10.

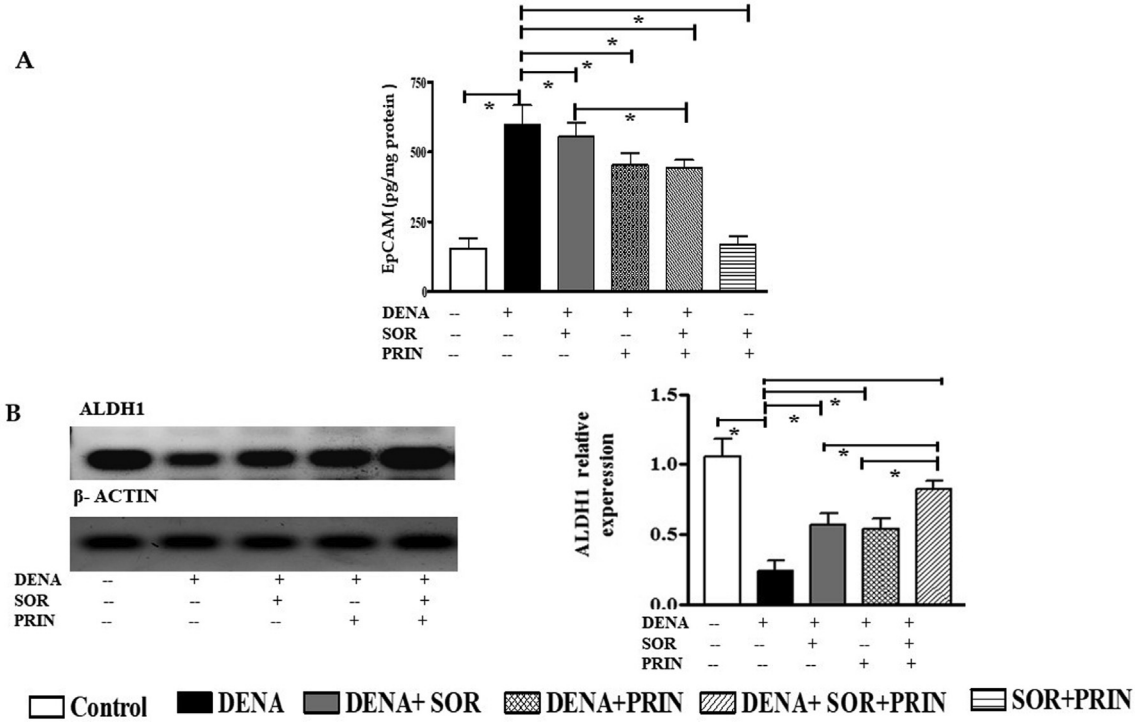

Fig. 5. Perindopril **downregulates EpCAM expression.** Fig. 5 illustrates the effect of sorafenib, Perindopril, and combination of them on EpCAM (Fig. 5 A) and ALDH (Fig. 5B). Data are expressed as means ± SD. * Value significantly *($p \leq 0.001$)* different between groups. n = 10.

Our results showed a significant hepatoprotective effect of perindopril whether used alone or combined with sorafenib. The latter showed a little but significant hepatoprotective activity when compared to DENA. Different studies reported potent inhibitory action of sorafenib or perindopril against ALT and AST serum levels in experimental animals with induced HCC (Ji et al., 2014;

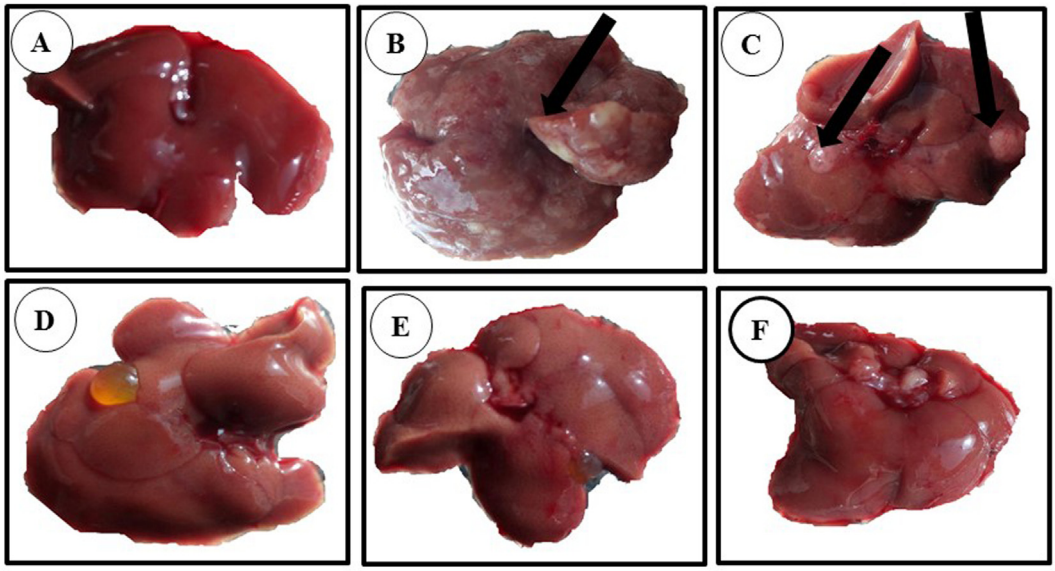

Fig. 6. Gross pathological examination of livers: Livers from mice in each group were examined macroscopically. liver from control mice (Fig. 6 **A**). Livers from DENA treated mice (Fig. 6**B&C**). Livers from mice treated with perindopril, sorafenib, or combination of both (Fig. 6 **D, E&F).**

Noguchi et al., 2003; Yamashita et al., 2009). Our study showed that the addition of perindopril to sorafenib exerted an additive hepatoprotective activity compared to each drug alone.

The antineoplastic effect of perindopril, sorafenib combination therapy was detected by histopathological examination of tissues where liver tissues from mice treated with the combination treatment protocol showed preserved architecture with some lymphocytes infiltration in portal tract and interface hepatitis but no dysplastic changes. The anticancer effect was further confirmed by observing the effect of this combination therapy on cancer characteristics such as angiogenesis and metastasis. Our results revealed that Perindopril or sorafenib reduced the expression of VEGF and MMP-9 in liver tissues. Moreover, combining the two drugs showed enhanced potency in dampening the expression of these proteins compared to each drug alone.

It has been reported that Perindopril could target the angiogenesis pathway and retard metastasis in experimental HCC models (Ji et al., 2014; Noguchi et al., 2003) similarly, sorafenib the multikinase inhibitor is associated with inhibition of tumor cell proliferation, angiogenesis, and metastasis (Nasr, Selima, Hamed, & Kazem, 2014; Zhu, Zheng, Wang, & Chen, 2017).

These results were in agreement with different studies that report the antineoplastic effect of ACE inhibitors. Enalapril as one of the ACE inhibitors has been reported as a chemosensitizer to 5-fluorouracil in the colorectal tumor through inhibition of NF-κB/STAT3-regulated proteins (Yang et al., 2020). Perindopril as well potentiated the antineoplastic effect of interferon-beta on HCC by decreasing vascularization of the tumor and inhibiting VEGF (Noguchi et al., 2003).

We try to shed a light on the possible relation between the observed chemosensitizing effect of perindopril and the presence of LCSCs through investigating different signaling pathways that maintain its phenotyping. We hypothesize that perindopril/sorafenib combination potentially inhibits Wnt/β-catenin pathway. The activity of this pathway started by the Binding of Wnt proteins with frizzled receptors and the LRP co-receptor. This receptor is a G-protein coupled receptor that is associated with a tertiary complex formed by axin, APC (adenomatous polyposis coli protein), CK1α, and GSK 3β (glycogen synthase kinase-3β (GSK-3β). Wnt receptor interaction results in inactivation of GSK 3β. The inactivated GSK 3β cannot phosphorylate the downstream molecule β-catenin this results in the stabilization of β-catenin allowing it to bind with its transcription sequence in the nucleus. (Pai et al., 2017) (**Fig-8**).

This signaling pathway potentially directs HCC cells proliferation, metastasis as well as drug resistance capacity. It plays an important role in maintaining the stemness characteristics of LCSCs through the regulation of Notch1. Recently, It has been reported that inhibitor to Wnt /β-catenin may be a potential target for HCC treatment (W. Wang et al., 2019; Zhang et al., 2016) Wnt / β-catenin signals also regulates cell cycle during proliferation. It has been reported that this pathway decreases the expression of cyclin D1 which plays an important regulatory role in the progression of the cells into the proliferative stage of the cell cycle (W. Wang et al., 2019). These data were consistent with our results where the proposed combination therapy in our study inhibited the expression of Wnt /β-catenin and consequently dampened expression of cyclin D1.

The effect of perindopril on Wnt /β-catenin pathway may be due to its main mechanism of action as an ACE inhibitor. The crosstalk between the renin-angiotensin system and Wnt/β-catenin signaling has been evidenced in different diseases such as chronic kidney diseases (Zhou & Liu, 2016). Up to our knowledge, this is the first study that demonstrates the potential activity of perindopril as a Wnt /β-catenin inhibitor in HCC.

The effect of perindopril on Wnt /β-catenin pathway may be also a consequence of its effect on leptin expression where our results reported that perindopril but not sorafenib inhibits leptin expression in liver tissues. The data were consistent with previous studies that confirm the role of perindopril in induction of leptin serum level (Mathai et al., 2008). Leptin and Wnt /β-catenin interaction has been examined previously where Leptin signaling pathway is associated with activating of PI3K-Akt. The phosphorylated Akt can phosphorylate the downstream molecule glycogen synthase kinase-3β (GSK-3β) which result in its inactivation (Yu et al., 2019) Fig. 8. GSK-3β inactivation enables β-catenin stabilization and allows it to bind with its transcription sequence in the nucleus thus activating the Wnt / β-catenin signaling (Pai et al., 2017).

In addition to leptin - Wnt / β-catenin interaction this hormone is a mitogenic and pro-angiogenic factor that induces other oncogenic pathways that modulate cancer stem cell proliferation such

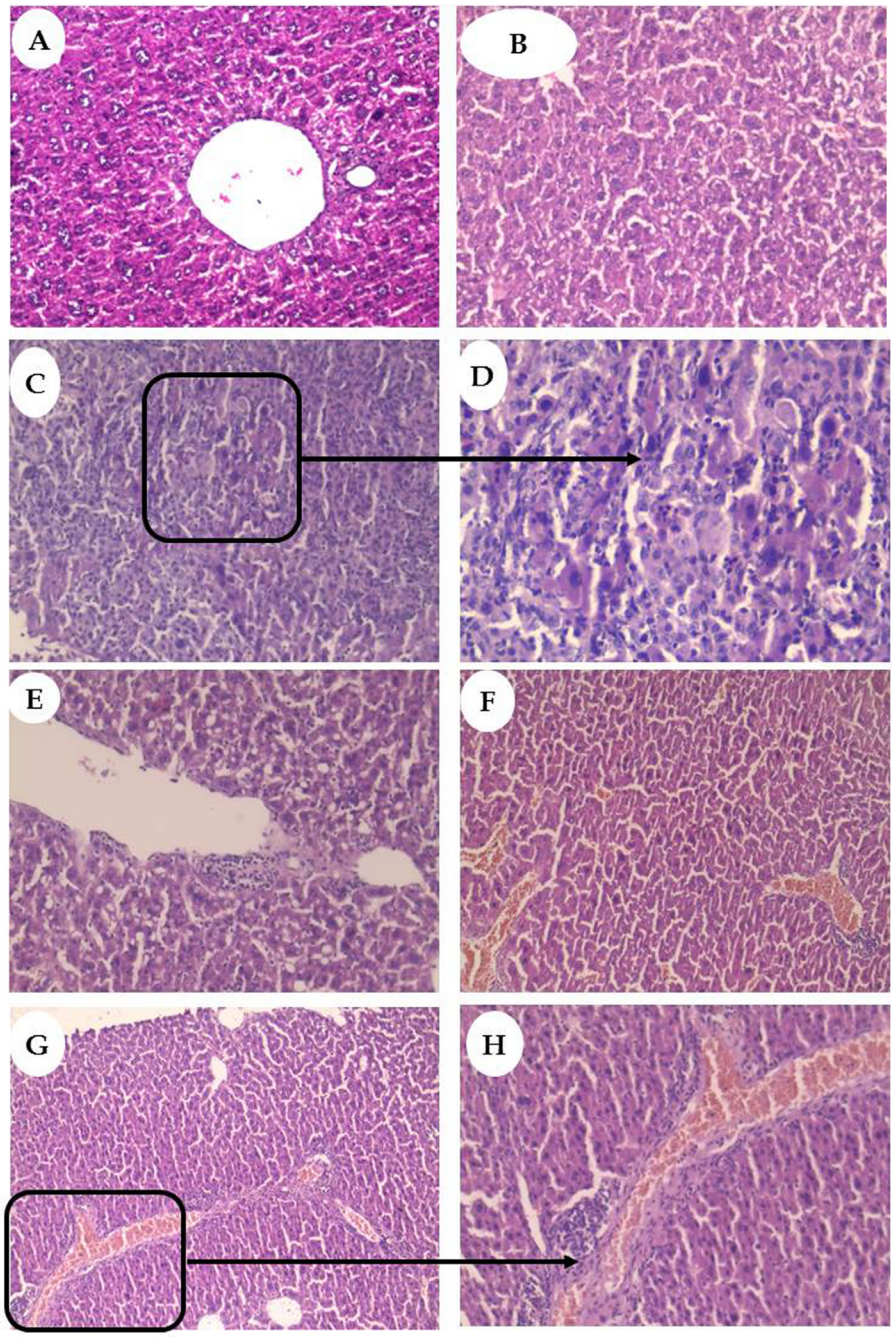

Fig. 7. Histopathological examination of liver tissues: Liver tissues were processed and stained with hematoxylin and eosin. Control as well as sorafenib and perindopril treated mice (without DENA) showed normal hepatic architecture (Fig. 6**A-B**). On the other hand, liver sections from DENA treated mice displayed multiple nodules in liver tissue. Each nodule showed pleomorphic hepatocytes arranged in trabeculae (more than two cells thick. Focal spindling could be seen. **(**Fig. 6**C-D).** The effect of sorafenib on liver tissue is represented in **(**Fig. 6**E)** where liver sections showed macrovesicular steatosis (mild steatosis) with a focal small area of hepatocyte degeneration. Preventative liver section from perindopril treated mice showed preserved normal liver architecture with moderate infiltration by lymphocytes in some portal tracts and nuclear dysplasia in the form of hyperchromatic nuclei (Fig. 6**F**). Liver sections from mice treated with combination therapy Sorafenib and Perindopril showed normal liver architecture with some lymphocytes infiltration in portal tract and interface hepatitis but no dysplastic changes were seen (Fig. 6**G-H)**. The images shown are representative photomicrographs. Magnification in F, G 100x, A, B, E, C H 200x and D 400x.

as Notch (Bender, Sifuentes, & Denver, 2017; Lipsey, Harbuzariu, Daley-Brown, & Gonzalez-Perez, 2016).

Hence, our results suggest that perindopril sensitizes HCC to sorafenib through damping the signaling axis that activates LCSC progression such as leptin /Wnt /β catenin pathway.

For further confirmation of the correlation between perindopril and LCSCs, we investigated the expression of EpCAM (epithelial cell adhesion molecule) a marker of liver cancer stem cell (Yamashita et al., 2009), and ALDH1, the enzyme that has been linked to stem cell and cancer stem cell population Tomita et al., 2017. Our results revealed that ALDH1 expression was induced by perindopril or sorafenib. Moreover, the combination of the two agents significantly increased ALDH1 expression compared to each drug alone. It has been reported that ALDH1 expression was associated with a good prognosis in HCC patients. It was demonstrated that ALDH1 expression is detected by immunohistochemistry in well-differentiated hepatocytes, not CSCs (Tanaka et al., 2015; Tomita H).

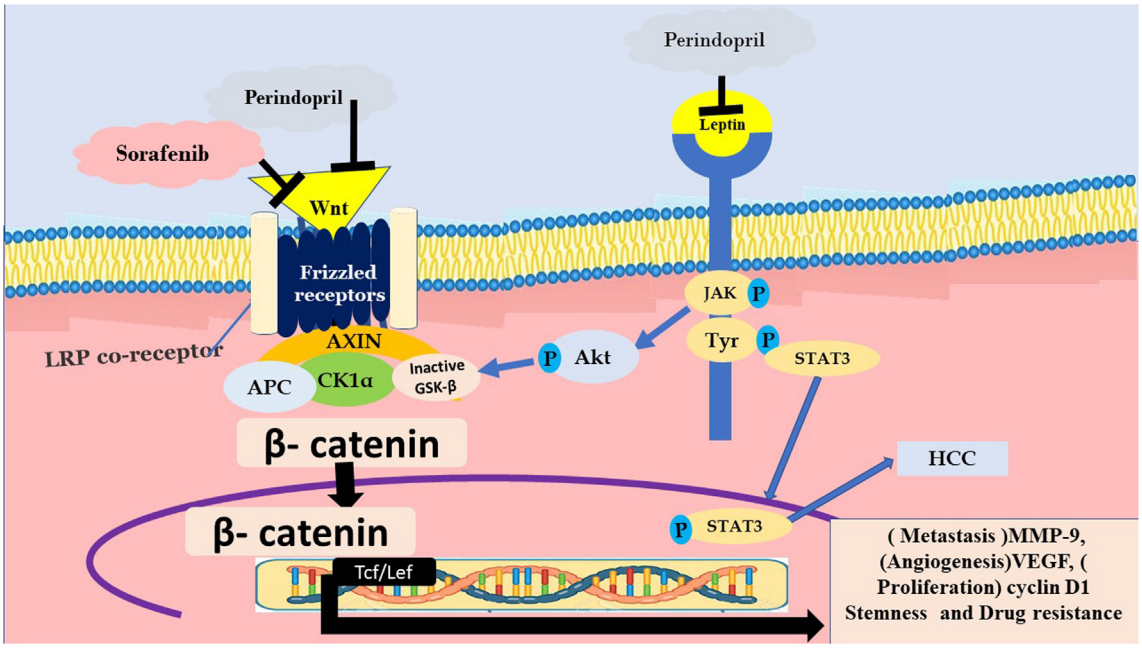

Fig. 8. Schematic diagram of molecular targets of sorafenib and perindopril.sorafenib and perindopril inhibit Wnt/ β-catenin pathway whereas perindopril potentiates this inhibition through dampening leptin level. Leptin is associated with Akt phosphorylation and inactivation of GSK 3β (more details in text).

In contrast to our results, ALDH1 was found to colocalize with different markers of cancer stem cells such as CD 133 (CD133), EpCAM (epithelial cell adhesion molecule), CD44, CD24, CD90, oval cell antigen-6 (OV6) in hepatobiliary tumors (Tomita H). However, we should note that cancer stem cells may express genes of normal stem cells (Tanaka et al., 2015).

On the other hand, our results demonstrated that perindopril whether used as a single therapy or a chemosensitizer to sorafenib treatment exerted downregulation of EpCAM expression whereas sorafenib results in non-significant changes in EpCAM expression. The effect of sorafenib on the expression of LCSCs markers is controversial where (Guan et al., 2015) reported that sorafenib increased positive EpCAM tumor cells such as Huh-7 which may be responsible for growth of new tumors. on the hand, another study demonstrated that sorafenib was able to suppress HCC metastasis through inhibition of communication between EpCAM positive cells and dampened expression of CD90 positive cells (Yoshida et al., 2017).

The inhibitory effect of perindopril on EpCAM expression may be due to its major mechanism of action as ACE inhibitors where the renin-angiotensin system has a pivotal role in the differentiation and maintenance of cancer stem cells (Roth, Wickremesekera, Wickremesekera, Davis, & Tan, 2019) also this inhibitory effect on LCSCs may be consequent to perindopril effect of leptin / Wnt /β catenin signaling where this pathway is highly upregulated in LCSCs (Pandit et al., 2018).

In conclusion, perindopril can be a useful chemosensitizing agent for sorafenib. The ability of the drug to decrease chemoresistance was associated with the dampened expression of leptin /Wnt /β-catenin axis as well as inhibition of liver cancer stem cells growth.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

Alaa El-sisi proposed the idea, supervised the practical work and reviewed the final manuscript. Sherin Zakaria shared in idea conformation, designed the experimental work, performed the in-vivo study, shared in the biochemical part of the research, analyzed the data, and wrote the final manuscript. Shady Allam performed the biochemical part of the study.

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Ethical approval

The experimental procedures were done in compliance with the ARRIVE guidelines in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The Ethics Committee of Kaferelsheikh University has approved the study protocol including the use of animals during the experiment.

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