TURKISH VETERINARY JOURNAL

Research Article

Anticancerogenic Effects of Silymarin on Diethylnitrosamine-Induced Hepatocellular Carcinoma in Wistar Rats

Emin Karakurt*, Serpil Dağ

Kafkas Üniversitesi, Veteriner Fakültesi, Patoloji Anabilim Dalı, Kars, Türkiye

Karakurt E 0000-0003-2019-3690 Dağ S 0000-0001-7667-689X

*Corresponding Author's E-Mail: mehmeteminkarakurt@hotmail.com

Abstract

The aim of this study was to evaluate the anticancerogenic effect of Silimarin by immunohistochemical methods on Diethylnitrosamine (DEN) induced Hepatocellular carcinoma (HCC) model in Wistar rats. Fifty male Wistar-Albino rats aging 2 months were randomly divided into 5 equal groups. The Control group rats were given pellet feed and drinking water for 20 weeks. The Sham group rats were injected intraperitoneally Propylene glycol dissolved in %0.9 NaCl three times for 20 weeks. The Silymarin group rats were injected intraperitoneally 100 mg/kg Silymarin three times a week for 20 weeks. The DEN group rats were injected intraperitoneally 50 mg/kg DEN once a week for 20 weeks. The DEN + Silymarin group rats were injected 50 mg/kg DEN once a week for 20 weeks. Silymarin at a dose of 100 mg/kg was injected intraperitoneally for 21 weeks, three times a week, starting 1 week before DEN administration. At the end of 21 weeks, macroscopically multifocal nodular structures were detected in the DEN and DEN + Silymarin group. A mix pattern of trabecular and acinar structures of HCC was observed in DEN and DEN + Silymarin groups. Besides; No statistically significant results were found between DEN and DEN + Silymarin groups in terms of iNOS, Nitrotyrosine, PCNA, GSTPi, MMP-9, HPIP and TUNEL staining ($P > 0.05$). In line with the results of our study, we determined that Silymarin did not show the expected anticarcinogenic effect.

Received 25 December 2019
Received in revised form 17 March 2020
Accepted 11 May 2020

Key words:
Diethylnitrosamine, hepatocellular carcinoma, liver cancer, rats, silymarin

Cite this article: Karakurt E, Dağ S (2020) Anticancerogenic Effects of Silymarin on Diethylnitrosamine-Induced Hepatocellular Carcinoma in Wistar Rats. Turk Vet J, 2(1):7-16.

Wistar Ratlarında Dietilnitrozamin Uygulanarak Oluşturulan Hepatoselüler Karsinom Modelinde Silimarinin Antikanserojenik Etkileri

Özet

Bu çalışmada Wistar ratlarında Dietilnitrozamin (DEN) uygulaması yapılarak oluşturulan hepatoselüler karsinom (HCC) modelinde, Silimarin'in antikanserojenik etkileri immunohistokimyasal yöntemlerle araştırılması amaçlandı. 50 adet 2 aylık Wistar Albino cinsi erkek rat rastgele olacak şekilde 5 gruba ayrıldı. Kontrol grubundaki ratlara 20 hafta boyunca pelet yem ve içme suyu verildi. Sham grubundaki hayvanlara % 0.9 NaCl içerisinde çözdürülmüş propilen glikol intraperitoneal yolla haftada 3 kez olmak üzere 20 hafta boyunca verildi. DEN grubundaki hayvanlara 50 mg/kg dozunda haftada 1 kez olmak üzere 20 hafta boyunca DEN uygulaması yapıldı. DEN+Silimarin grubundaki ratlara DEN uygulamasından 1 hafta önce başlayacak şekilde 100 mg/kg dozunda haftada 3 kez olmak üzere 21 hafta boyunca intraperitoneal yolla Silimarin uygulandı. Silimarin grubundaki ratlara 100 mg/kg dozunda 20 hafta boyunca intraperitoneal yolla haftada 3 kez olmak üzere Silimarin uygulandı. 21 hafta sonunda DEN ve DEN+Silimarin gruplarında makroskobik olarak multifokal nodüler yapılar tespit edildi. Mikroskobik incelemeler sonucunda DEN ve DEN+Silimarin gruplarında HCC'nin trabeküler ve asiner paternini içeren miks tipi tespit edildi. iNOS, Nitrotirozin, PCNA, GSTPi, MMP-9, HPIP ve TUNEL boyamaları sonucunda DEN ve DEN+Silimarin grupları arasında istatistiki karşılaştırmada anlamlı sonuç bulunamadı ($P > 0.05$). Çalışmamızın bulguları doğrultusunda Silymarin'in beklenen antikanserojenik etkiyi göstermediğini tespit ettik.

Anahtar kelimeler: *Dietilnitrozamin, hepatoselüler karsinom, karaciğer kanseri, ratlar, silimarin.*

Introduction

Hepatocellular carcinoma (HCC) constitutes 70-85% of the primary malignant tumors of the liver (Shirakami et al. 2012). It is ranked fifth in cancer cases worldwide and ranks third in cancer-related causes of death (Medhat et al. 2017).

Diethylnitrosamine (DEN), an N-Nitrozo alkyl component, is used as an initiating agent in two-step (initiation and promotion) hepatocarcinogenic study protocols. Carcinogenic effect of DEN; It is possible to hydroxylate with cytochrome p450 enzymes and convert them to bioactive with the alkylation mechanism (Bingül et al. 2013). The intermediate

products formed after bioactivity (06-ethyl deoxy guanosine and 04- to 06-ethyl deoxy thymidine) exhibit low affinity to the binding sites of various enzymes, thus producing covalent bonds with important compounds of the cells instead of urinary excretion. As a result, they cause mutations, necrosis and cancer (Merhan et al. 2016). The bioactivation process is an important step to initiate carcinogenesis (Bingül et al. 2013). DEN, which becomes bioactive, reacts with the DNA and causes the ethylation of the bases. Ethyl DNA adducts interrupt base pairs, leading to mutations, activation of protoncogenes (eg RAS) and inhibition of tumor suppressor genes (eg p53). This often results in HCC (Matsuda et al. 2005).

Silybum marianum is an annual or biennial herbaceous plant belonging to the family *Asteraceae* (Bosch-Barrera & Menendez 2015). Seeds of *Silybum marianum* include approximately 70-80% Silymarin. The remaining and approximately 20-30% of the part is not chemically defined. Silymarin is a mixture of four flavonolignan isomers called Silybin, Isosilybin, Silydianin and Silycristin. The ratio of isomers; Silybin 60-70%, Silycristin 20%, Silydianin 10% and Isosilybin 5% (Ashraf et al. 2015). The researchers identified ten important characteristics of cancer. These are uncontrolled growth, resistance to death, replicative immortality, induction of angiogenesis, invasion, metastasis, genomic instability, tumor inflammation, escape from immune system and re-programming of energy metabolism. It has been reported in recent studies that Silymarin and Silybin have a healing effect on breast, skin, colon, cervix, ovarian, prostate, lung and liver cancers (Fan et al. 2014; Ben Rahal et al. 2015). Research shows that Silymarin is capable of affecting all of the above-mentioned characteristics in cancers observed in humans (Bosch-Barrera & Menendez 2015). Silymarin and / or Silibinin exhibit anticarcinogenic effect by regulating the cell cycle, inducing apoptosis, and different mechanisms such as inhibition of angiogenesis, invasion and metastasis, and inflammation suppression (Eo et al. 2015). In this study, Silymarin's anticancer effect on cell proliferation, metastasis, transformation capacity, oncogenes and apoptosis was evaluated by various immunohistochemical markers in the hepatocellular carcinoma model inducing by DEN.

Materials and Methods

Animals and ethics committee

Fifty male Wistar-Albino rats aging 2 months were provided by Erzurum Veterinary Control Institute Experimental Animals Unit. The rats were housed under suitable conditions (23 ± 2 °C, % 55 humidity rate, 12-hour day-night illuminating cycle) in the Kafkas University Experimental Animals In the Application and Research Center Laboratory. During the trial, the animals were given standart feed and water ad libitum. Prior to the experiment, the rats were prepared for manipulation during a 1 week habituation period. The ethics committee report of the study was taken from Kafkas University Animal Experimentals Local Ethics Committee (KAU-HADYEK-2015-095).

Experimental design

The rats were randomly divided into 5 equal groups. The Control group rats were given pellet feed and drinking water for 20 weeks. The Sham group rats were injected intraperitoneally Propylene glycol (75/25) dissolved in 0.9% NaCl three times for 20 weeks. The Silymarin group rats were injected intraperitoneally 100 mg/kg Silymarin (Sigma-Aldrich, N 0292) three times a week for 20 weeks. The DEN group rats were injected intraperitoneally 50 mg/kg DEN (Sigma N 0756) once a week for 20 weeks. The DEN + Silymarin group rats were injected 50 mg/kg DEN once a week for 20 weeks. Silymarin at a dose of 100 mg/kg was injected intraperitoneally for 21 weeks, three times a week, starting 1 week before DEN administration.

At the end of the administration, the animals were euthanized by pentobarbital. The livers of all rats with systemic necropsies were examined for macroscopic changes and tissue samples were taken for histopathological-immunohistochemical examinations.

Histopathological, immunohistochemical and TUNEL methods

Liver tissue samples were fixed in 10 % buffered formaldehyde solution (Merck). After routine procedures, paraffin blocks were prepared and sections with a thickness of 5 μm were taken for Hematoxylin and Eosin (H&E) staining. Sections were examined with H&E in the light microscope (Olympus Bx53) to determine the histopathological changes and photographed with Cell ^P Program (Olympus Soft Imaging Solutions GmbH, 3,4).

Avidin-Biotin Peroxidase method was used as immunohistochemical stain. For immunohistochemical staining, sections of 4 μm thick from paraffin blocks were rehydrated. In order to prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide

solution for 15 minutes. Then, the microwave method was applied to the sections to reveal the antigenic receptors (Citrat Buffer Solution pH 6 for 25 min). In order to prevent nonspecific staining, the sections were incubated for 30 min with non-immune serum (Genemed Biotechnologies REF 54-0003). Following treatment with Phosphate Buffered Salt Solution (PBS) with different proportions of diluted antibodies (PCNA (Santa Cruz, Sc-56, Dilution Ratio 1: 100), GSTPi (Novocastra, NCL-L-GSTPi-438, Dilution Ratio 1: 100), MMP-9 (Santa Cruz, Sc-393859, Dilution Ratio 1: 100) and HPIP (Bethyl Lab, IHC-00327, Dilution Ratio 1: 100) were incubated for one hour. The sections were washed 3 times in PBS solution for 5 minutes, and the biotinised secondary antibody (Genemed Biotechnologies REF 54-0003) were applied to them at room temperature for 30 minutes. After washing in PBS (3-5 min), all sections were incubated with peroxidase-bound Strep Avidin (Genemed Biotechnologies REF 54-0003) for 30 minutes. A solution of 3.3-diaminobenzidine tetra hydrochloride (DAB) (Genemed Biotechnologies REF 10-0048) were used as colour revealing substrate. The sections were stained with Mayer Hematoxylin and coated with immune mount.

TUNEL method was used to detect apoptotic cells and the In Situ Cell Death Detection Kit, POD (Roche 11684817910) was used for this purpose. Sections were kept in the incubator at 37°C overnight and passed through xylene solution. Later sections were rehydrated with a decreasing percentage of alcohol series and washed with distilled water. Subsequently, sections were incubated with proteinase K solution (Sigma) at 37 ° C for 25 minutes. The sections were then washed 3 times with PBS solution for 3 minutes. After washing with PBS, 3% hydrogen peroxide solution was applied to the sections for 15 minutes to block the endogenous peroxidase activity. Sections were washed again with PBS and incubated with TUNEL solution (Roche 11684817910) at 37 ° C for 60 minutes. The tissues were then washed 3 times with PBS for 3 minutes. At follow-up, the sections were incubated with Peroxidase (POD) solution (Roche 11684817910) for 30 min at 37 ° C. Sections were then again washed 3 times with PBS for 3 minutes. After washing, DAB solution (Genemed Biotechnologies) was dropped and stained with Mayer Hematoxylin. The sections were covered with entallen after passing through an increasing percentage of alcohol and xylol solution. The slides prepared after the covering were examined under a light microscope (Olympos Bx53) and photographed via the Cell^P program (Olympos Soft Imaging Solutions Gmbh, 3,4). Analyzes of the images were done with Image J Program (1.51j8). Analysis of immunohistochemical and TUNEL staining results were performed by a grading system based on the number of positive cells in the areas that best reflect the character of staining. For quantification of the staining in the tissue, the analysis was started on the basis of high intensity reaction areas. For each sample, 10 different areas were examined at a total enlargement of 200. The number of cells stained positively in each area was recorded and the average of these 10 sites was taken as the data of that animal.

Statistical analyses

Shapiro-Wilk test was used to evaluate the normal distribution of the groups. T-test was used for the evaluation of groups with normal distribution and Mann-Whitney-U test was used for the evaluation of groups that didn't show normal distribution. Statistical Package for Social Sciences (SPSS) 20 Program was used in statistical tests.

Results

Macroscopic results

No lesions were found in the livers of rats in Control (Figure 1a), Sham (Figure 1b) and Silymarin groups (Figure 1c). Multifocal nodules and hemorrhagic areas were observed in the liver of rats in the DEN group (Figure 1d). In rats in the DEN + Silymarin group (Figure 1e), nodular structures similar to the rats in the DEN group were observed.

Hematoxylin & eosin results

We observed that liver tissue retained its normal structure in histopathological examination of livers Control (Figure 2a), Sham (Figure 2b) and Silymarin (Figure 2c). HCC was observed in the rats of the DEN group. Trabecular and acinar pattern were dominant in tumor tissue. In some areas only trabecular structures were observed, whereas in some areas only acinar structures were found. There were also areas where these two patterns were observed as mixed (Figure 2d). HCC was also diagnosed in animals administered with DEN Silymarin. Similar to the DEN group, the common histopathological appearance was a mix pattern with trabecular and acinar structures (Figure 2e).

Table 1. Average PCNA, GSTPi, MMP-9, HPIP and TUNELpositive cell numbers for all groups

Groups	Group 1 (Control) n = 10	Group 2 (Sham) n = 10	Group 3 (Silymarin) n = 10	Group 4 (DEN) n = 10	Group 5 (DEN + Silymarin) n = 10
PCNA	---*	---*	---*	82.9 ± 6.17 [a]	79.8 ± 6.74 [a]
GSTPi	---*	---*	---*	176,1 ± 7.52 [a]	154.3 ± 11.54 [a]
MMP-9	---*	---*	---*	32.5 ± 4.84 [a]	29.5 ± 3.21 [a]
HPIP	---*	---*	---*	60.5 ± 7.46 [a]	50 ± 5.45 [a]
TUNEL	---*	---*	---*	51.6 ± 5.27 [a]	59.8 ± 6.77 [a]

* is equal to zero.

[a]There is no statistically difference between DEN and DEN + Silymarin groups (P > 0.05).

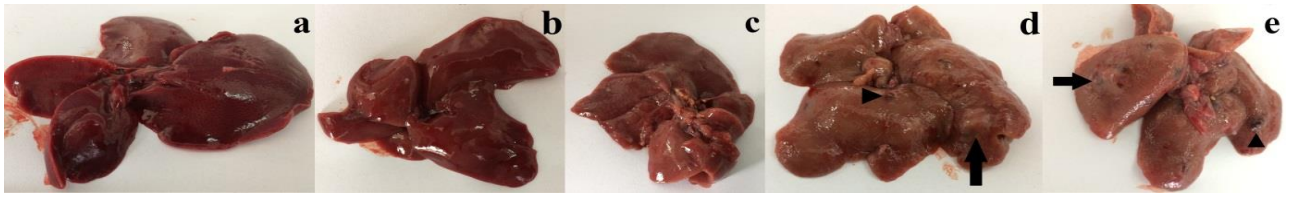

Figure 1. Macroscopic appearance of the livers from control and experimental group of rats. Control Group (a), Sham Group (b), Silymarin Group (c), DEN Group, HCC nodule (arrow) and hemorrhage (arrowhead) (d) DEN + Silymarin Group, HCC nodule (arrow) and hemorrhage (arrowhead) (e).

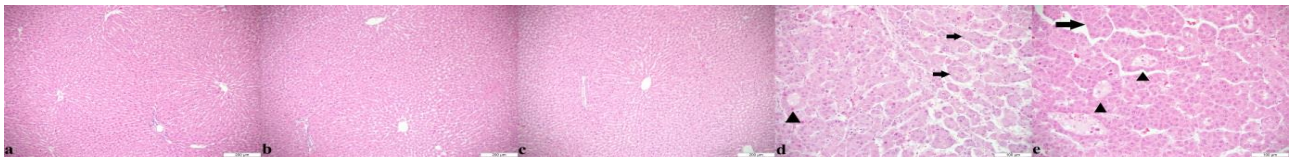

Figure 2. Histopathologic appearance of the livers from control and experimental group of rats. Control Group (a), Sham Group (b), Silymarin Group (c), DEN Group, trabecular (arrows) and glandular (arrowhead) structures (d) DEN + Silymarin group, trabecular (arrow) and glandular structures (arrowheads) (e), H&E, scale bar = 100 μm.

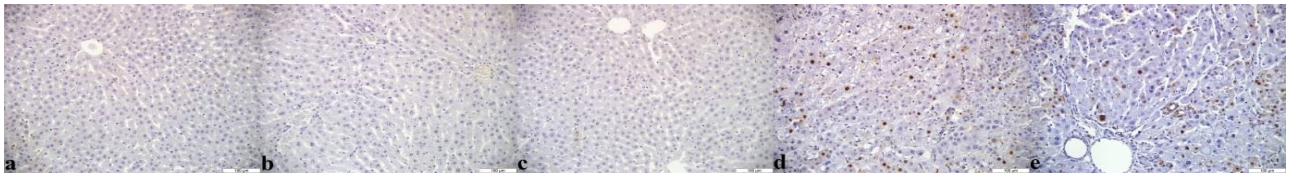

Figure 3. The liver sections from control and experimental group of rats. Control Group (a), Sham Group (b), Silymarin Group (c), DEN Group, positive immune reaction in hepatocyte nuclei (d), DEN + Silymarin, positive immune reaction in hepatocyte nuclei (e), PCNA stain, scale bar = 100 μm.

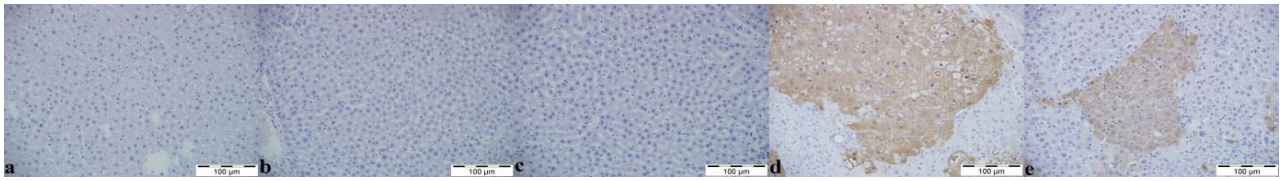

Figure 4. The liver sections from control and experimental group of rats. Control Group (a), Sham Group (b), Silymarin Group (c), DEN Group, brown immune positive reaction in transformed area (d), DEN + Silymarin, brown immune positive reaction in transformed area (e), GSTPi stain, scale bar = 100 μm.

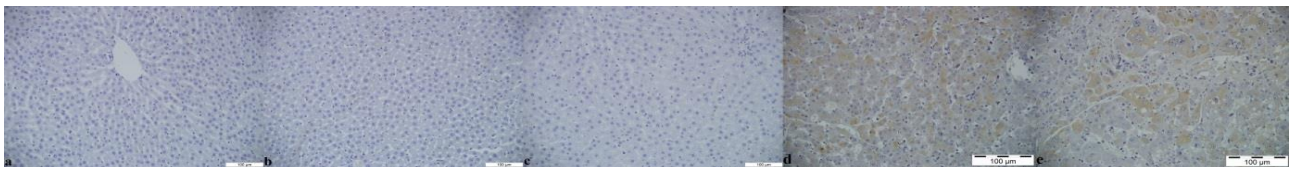

Figure 5. The liver sections from control and experimental group of rats. Control Group (a), Sham Group (b), Silymarin Group (c), DEN Group, intracytoplasmic immune reaction (d), DEN + Silymarin, intracytoplasmic immune reaction (e), MMP-9 stain, scale bar = 100 μm.

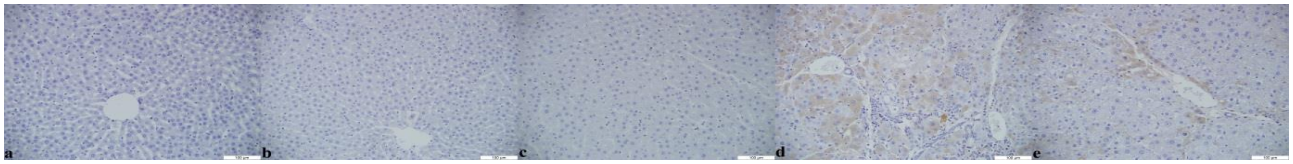

Figure 6. The liver sections from control and experimental group of rats. Control Group (a), Sham Group (b), Silymarin Group (c), DEN Group, expression in the cytoplasm of hepatocytes (d), DEN + Silymarin, expression in the cytoplasm of hepatocytes (e), HPIP stain, scale bar = 100 μm.

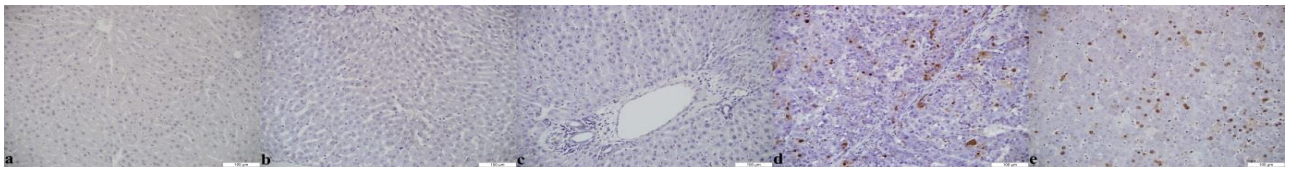

Figure 7. The liver sections from control and experimental group of rats. Control Group (a), Sham Group (b), Silymarin Group (c), DEN Group, positive reaction in nuclei of hepatocytes (d), DEN + Silymarin, positive reaction in nuclei of hepatocytes (e), TUNEL stain, scale bar = 100 μm.

Immunohistochemical results

The mean values of PCNA, GSTPi, MMP-9, HPIP and TUNEL positive cell numbers of all groups were given in Table 1. Control (Figure 3a), Sham (Figure 3b) and Silymarin (Figure 3c) groups were not positive for PCNA immune positive reaction. PCNA positive staining was observed in the nuclei of hepatocytes in DEN (Figure 3d) and DEN + Silymarin (Figure 3e) groups.

The mean number of positive stained cells in DEN group compared to DEN + Silymarin group was high but the difference between them was not statistically significant ($P > 0.05$) GSTPi expression was not observed in Control (Figure 4a), Sham (Figure 4b) and Silymarin (Figure 4c) groups, while immunoreactivity was found in DEN (Figure 4d) and DEN + Silymarin (Figure 4e) groups.

In the DEN and DEN + Silymarin groups, the brown GSTPi positive reaction was found particularly in large nodular structures. GSTPi expression was detected in both cytoplasm and nucleus of the hepatocytes forming these nodular structures. In the DEN group, the mean number of positive cells was 176.1 ± 7.52 and in the DEN + Silimarin group this number decreased to 154.3 ± 11.54. However, we found that this decrease was not statistically significant ($P > 0.05$).

MMP-9 immunoreactivity was not detected in Control (Figure 5a), Sham (Figure 5b) and Silymarin (Figure 5c) groups. MMP-9 immune positive staining in DEN (Figure 5d) and DEN + Silymarin (Figure 5e) groups was determined in the cell cytoplasm. Although there was a decrease in the mean values of MMP-9 positive cells in DEN + Silymarin group compared to DEN group, this decrease was not statistically dramatic ($P > 0.05$).

Control (Figure 6a), Sham (Figure 6b) and Silymarin (Figure 6c) groups were not positive for HPIP expression. HPIP expression was observed in cytoplasm of hepatocytes in DEN (Figure 6d) and DEN + Silymarin (Figure 6e) groups. While the mean value of HPIP positive cells increased in DEN group, it was determined that the administration of Silymarin decreased the number of HPIP positive cells but this decrease was not statistically important ($P > 0.05$).

TUNEL results

TUNEL positive staining was not observed in groups Control (Figure 7a), Sham (Figure 7b) and Silymarin (Figure 7c). TUNEL reaction in hepatocyte nuclei was determined in DEN (Figure 7d) and DEN + Silymarin (Figure 7e) groups.

Although it was observed that Silymarin administration increased the mean value of TUNEL positive cell compared to DEN group, this increase was not statistically significant ($P > 0.05$). Except for the brown stained nuclei positive cells, both cytoplasmic and nuclear staining cells were observed in DEN and DEN + Silymarin groups. These cells were considered necrotic and not subjected to statistical evaluation.

Discussion

Detection of cell proliferation rate is particularly important in diseases such as cancer (Srigopalram et al. 2014). PCNA is a 36 kDa nuclear protein that plays an important role in the replication, recombination and repair of genomic DNA (Taha et al. 2010). PCNA is correlated with DNA synthesis and cell proliferation rate (Abdel Salam et al. 2007). Detection of PCNA by immunohistochemical methods indicates both DNA replication and DNA damage resulting in carcinogenesis (Abike & Zengeroğlu 2010). In our study, we aimed to determine the uncontrolled cell proliferation of liver tissue and malignant transformation by evaluating PCNA immunohistochemically.

In the studies of liver cancer [11, 15-17] induced by DEN, it was shown that DEN administration increased PCNA expression compared to control groups. In our study, we found that DEN administration caused a significant increase in the number of PCNA positive cells compared to the control group in a similar way to the literature data (Raghunandhakumar et al. 2013; Song et al. 2013; Srigopalram et al. 2014; Zhao et al. 2014). We observed PCNA expression in the nucleus of hepatocytes. We interpreted this increase in the number of PCNA positive cells to the capacity of DEN administration to mutate and generate DNA damage.

In the studies of experimentally induced HCC (Gopalakrishnan et al. 2013), it was demonstrated that treatment of Silymarin significantly reduced the expression of PCNA and showed antiproliferative effect by immunohistochemically. In this study, contrast to the literature data (Gopalakrishnan et al. 2013), we observed that the treatment of Silymarin resulted in a decrease in PCNA positive cell number. However, there was no statistically dramatic difference between DEN and DEN + Silymarin groups. We interpreted this result as Silymarin showing partial antiproliferative effect.

GSTs are involved in the detoxification of carcinogenic or mutagenic substances. GSTs are divided into three main groups: (α) basic, (π) acidic, (u) neutral. GSTPi is considered as a potential tumor marker in hepatomas, gastric carcinomas and colonic carcinomas. In addition, GST levels were found to be high in patients with primary HCC and GST measurements were reported to be a usable marker for HCC (Aköz et al. 2010). In our study, we aimed to determine the preneoplastic and dysplastic foci that play an important role in HCC development by evaluating GSTPi expression immunohistochemically.

In liver cancer model studies (Shizuma et al. 2011; Fujise et al. 2012; Thumvijit et al. 2014; Abdo et al. 2015), researchers determined that DEN administration increases GSTPi expression compared to the control group especially in cellular transformation regions. They observed that GSTPi positive areas increased significantly in cancer groups both in terms of cell numbers and area measurements compared to the control group. In our study, we observed that GSTPi immunopositive areas increased significantly both in number and size in the DEN group compared to the control group in accordance with the literature data (Shizuma et al. 2011; Fujise et al. 2012; Thumvijit et al. 2014; Abdo et al. 2015). Although, we observed that GSTPi expressions were more severe in the transformed hepatocyte focuses that formed the nodular structures in particular. We interpreted this increase in expression that DEN administration effectively initiated the process of carcinogenesis in liver tissue.

Imamoto et al. (2014), induced liver cancer by administration DEN for 12 and 18 weeks. In the protective groups treated with Silymarin, they showed that the expression levels of GSTPi decreased compared to the DEN group but this decrease was not statistically significant. In our study, in accordance with the literature data [30], the treatment of Silymarin was found to cause a decrease in GSTPi positive cell number. But this decrease was not statistically important.

MMPs play a critical role in extracellular matrix (ECM) detoriation, metastasis, tumor cell invasion and angiogenesis (Mansour et al. 2014). MMP-2 and MMP-9 are the most detected matrix metalloproteinases in malignant tissues (Ramakrishnan et al. 2009). They play a crucial role in the metastatic process by locally disintegrating the basement membrane and the extracellular matrix (Sivaramakrishnan et al. 2009; Wu et al. 2014). In our study, we evaluated the MMP-9 expression immunohistochemically in order to demonstrate the role it plays in HCC metastasis.

In previous studies, MMP-9 expression was investigated immunohistochemically in the liver (Subramaniyan et al. 2014) and lung cancer models (Man et al. 2015) In the DEN-induced cancer groups, MMP-9 expression was observed in the cytoplasm of tumor cells. MMP-9 expression was found to be higher in the rats in the DEN group compared to the rats in the control group. In our study, we found that DEN administration significantly increased the expression of MMP-9 compared to the control group in a similar way to the literature data (Subramaniyan et al. 2014; Man et al. 2015). In addition, we observed MMP-9 expression in the cytoplasm of hepatocytes in particular. We believed that this increase in expression was consistent with the detoriation of ECMs caused by DEN administration. As a result of the destruction of ECMs, the barrier to tumor invasion and migration was removed. The role of matrix metalloproteinases in removing this barrier is very important. This increase in the number of positive cells observed in the DEN group is also important in terms of the role of matrix metalloproteinases, especially MMP-9, in the development of HCC. As a matter of fact, we found a large number of metastatic foci originating from the liver in the lung of a rat in the DEN-administered group.

In the experimentally generated HCC (Ramakrishnan et al. 2009) and Ehrlich asites carcinoma (Beydogan & Bolkent 2016) model studies, MMP-9 expression was evaluated immunohistochemically in order to reveal the antimetastic effect of Silymarin and its main component, Silybin. Researchers found that these hepatoprotective agents significantly reduced the expression of MMP-9 compared to cancer groups. Incompatible with literature data (Ramakrishnan et al. 2009; Beydogan & Bolkent 2016), we determined that Silymarin treatment slightly reduced the number of MMP-9 positive cells. Although lung metastasis was seen in a rat in the DEN group, no lung metastasis was found in any rat in the DEN + Silymarin group. According to the data of our study, Silimarin showed an antimetastatic effect even though it was statistically insignificant. We concluded that Silymarin's down-regulation through MMP-9 could play an important role in this protective effect.

HPIP is a recently described oncogene, which is reported to be expressed extremely in cancer types such as non-small cell lung cancer, HCC, spinal glioblastoma, thyroid cancer, gastric cancer, colorectal cancer, breast infiltrative ductal carcinoma, oral carcinoma and melanoma (Chen et al. 2016; Bugide et al. 2017). In this study, we evaluated the expression of HPIP immunohistochemically so we aimed to determine the role of this oncogene in the development of experimentally induced HCC in rats. Thus, the link between inhibition of HPIP and the suppression of HCC development will also be demonstrated.

Xu et al. (2013a), reported that HPIP was over-expressed in most of 52 liver cancer patients. In another study Xu et al. (2013b) showed that HPIP levels were high in the majority of 328 liver cancer patients. Liver tissues of the control and cancer group were examined immunohistochemically and HPIP expression was significantly increased (> 90%) in liver cancer patients and immunoreactivity was observed especially in the cytoplasm. In the light of this information, HPIP is an oncogene that has an important role in the development and progression of liver cancer. It has been considered by researchers that blocking HPIP may be an effective strategy in the treatment of liver cancer. As a result of the inactivation of HPIP, they have demonstrated that tumor growth in the liver was regressed both in vivo and in vitro in genetically immune system blocked mice. In their study; HPIP was found to promote cell growth in HCC by activation of G2-M transition in the cell cycle. In our study, parallel to literature data (Xu et al. 2013b), we detected HPIP expression especially in the cytoplasm of hepatocytes in the HCC group that we formed by DEN administration.

There is no literature data on the experimental HCC model induced by DEN administration in rats and anticarcinogenic effects of Silymarin by immunohistochemical evaluation of HPIP expression. A significant increase in HPIP expression was observed in the DEN group compared to the control group. This increase was attributed to mutations resulting from DEN bioactivation. As previously reported (Matsuda et al. 2005), mutations resulting from DEN's bioactivation cause activations or inhibitions in important genes such as protooncogenes or tumor suppressor genes. HPIP, which is known to have important functions in cell proliferation, is basically an oncogene (Xu et al. 2013a). We thought that mutations resulting from the bioactivation of DEN could activate HPIP and trigger uncontrolled cell proliferation observed in HCC. In our study, although not statistically significant, we showed that Silymarin treatment caused a partial decrease in HPIP expression. We thought that this decrease may be related to the anti-proliferative capacity of Silymarin.

Apoptosis (programmed cell death) can be triggered by cancer, AIDS, oxidative stress diabetes and neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Elmore 2007; Coşkun & Özgür 2011; Eröz et al. 2012). We aimed of to determine the apoptotic cells in experimentally induced HCC progression and to demonstrate the proapoptotic capacity of Silymarin by the evaluation of TUNEL method in this study. Thus, it would be demonstrated that the development of HCC can be suppressed by triggering controlled cell death.

It has been demonstrated by several researchers that the number of TUNEL positive cells in experimental liver cancer model group induced by DEN is quite insignificant compared to control group (Xu 2011; Gupta et al. 2013; Vásquez-Garzón et al. 2013; Zhang et al. 2013). Contrary to these studies, there are studies showing significant increase in the number of TUNEL positive cells in DEN-induced liver cancer models compared to control groups (Bharati et al. 2014, Patial et al. 2015). Bharati et al. (2014), reported that the number of TUNEL-positive cells was significantly higher in the liver cancer model induced by DEN compare to control group. They observed that TUNEL positive staining was in the cytoplasm as well as in the nucleus. They identified the cells stained in this way as necrotic cells. In our study, similar to these studies (Bharati et al. 2014, Patial et al. 2015), we found that the number of TUNEL positive cells increased significantly in the DEN group compared to the control group. In addition, TUNEL positive reaction was observed in the cytoplasm as well as the nucleus of hepatocytes in the DEN group. Induction of apoptosis is important in the treatment of cancer (Kaya et al. 2012). It is known that cancer rid of apoptosis mechanism plays a pivotal role in carcinogenesis process (Eröz et al. 2012). Suppression of apoptosis is a driving force in cancer development and progression (Dinçel & Kul 2016). Death receptor CD95 expression, which plays an important role in induction of apoptosis, has been reported to decrease in tumor cells in HCC (Jin & El-Deiry 2005). Therefore, we expected that the number of TUNEL positive cells would be quite low in the DEN group. However, the number of TUNEL positive cells in the DEN group was significantly increased compared to control group. We interpreted this increase as a certain level of programmed cell death in HCC progression.

In previous studies, it has been determined that in the lung cancer (Wu et al. 2016) and prostate cancer (Deep et al. 2008), the TUNEL method has shown that Silymarin treatment increases the number of apoptotic cells compared to cancer groups and has a proapoptotic effect. Consistent with the literature data (Deep et al. 2008; Wu et al. 2016), we detected that Silymarin treatment increased the number of TUNEL positive cells and showed a mild proapoptotic effect compared to DEN group. However, we thought that the slight triggering of apoptosis caused by Silymarin treatment was not effective enough in the regression of HCC.

Conclusion

As a result, according to the data of our study, the administration of DEN once a week for 20 weeks was found to be very useful in inducing HCC model in rats. However, we observed that Silimarin, an important anticancerogenic agent, does not have a significant protective effect.

Acknowledgments

This study was supported by the Research Fund of Kafkas University (Project Number: 2016-TS-08, PhD thesis).

Conflict of Interest Statement

The authors declare no conflicts of interest with respect to the publication of this manuscript.

References

Abdel Salam OM, Sleem AA, Omara EA, Hassan NS (2007) Effect of ribavirin alone or combined with silymarin on carbon tetrachloride induced hepatic damage in rats. Drug Target Insights 2:19-27. https://doi.org/10.1177/117739280700200014

Abdo W, Hirata A, Shukry M, Kamal T, Abdel-Sattar E, Mahrous E, Yanai T (2015) Calligonum comosum extract inhibits diethylnitrosamine-induced hepatocarcinogenesis in rats. Oncol Lett 10:716–722. https://doi.org/10.3892/ol.2015.3313

Abike F, Zengeroğlu S (2010) Epitelyal over karsinomlarında PCNA ekspresyonu. Türk Hijyen ve Deneysel Biyoloji Dergisi 2010; 67:173-178.

Aköz M, Vatansev H, Gürbilek M, Akkuş İ, Vatansev C, Kaptanoğlu B (2000) Glutatyon S-transferaz (GST) izoenzimlerinin çeşitli kanser vakalarında araştırılması. Genel Tıp Derg 10: 1-6.

Ashraf M, Abid F, Riffat S, Bashir S, Iqbal J, Sarfraz M, Afzal A, Zaheer M (2015) Rationalized and complementary findings of silymarin (milk thistle) in Pakistani healthy volunteers. Acta Pol Pharm 72:607-614.

Ben Rahal N, Barba FJ, Barth D, Chevalot I (2015) Supercritical CO_2 extraction of oil, fatty acids and flavonolignans from milk thistle seeds: Evaluation of

their antioxidant and cytotoxic activities in Caco-2 cells. Food Chem Toxicol 83:275-282. https://doi.org/10.1016/j.fct.2015.07.006

Beydogan AB, Bolkent S (2016) The effects of silibin administration for different time periods on mouse liver with Ehrlich ascites carcinoma. Pharmacol Rep 68:543-549. https://doi.org/10.1016/j.pharep.2015.12.003

Bharati S, Rishi P, Koul A (2014) Azadirachta indica modulates electrical properties and type of cell death in NDEA-induced hepatic tumors. Cell Biochem Biophys 70:383-390. https://doi.org/10.1007/s12013-014-9923-6

Bingül İ, Başaran-Küçükgergin C, Tekkeşin MS, Olgaç V, Doğru-Abbasoğlu S, Uysal M (2013) Effect of blueberry pretreatment on diethylnitrosamine-induced oxidative stress and liver injury in rats. Environ Toxicol Pharmacol 36:529-538. . https://doi.org/10.1016/j.etap.2013.05.014

Bosch-Barrera J, Menendez JA (2015) Silibinin and STAT3: A natural way of targeting transcription factors for cancer therapy. Cancer Treat Rev 41:540-546. https://doi.org/ 10.1016/j.ctrv.2015.04.008

Bugide S, Gonugunta VK, Penugurti V, Malisetty VL, Vadlamudi RK, Manavathi B (2017) HPIP promotes epithelial-mesenchymal transition and cisplatin resistance in ovarian cancer cells through PI3K/AKT pathway activation. Cell Oncol 40:133-144. https://doi.org/ 10.1007/s13402-016-0308-2

Chen B, Zhao J, Zhang S, Zhang Y, Huang Z (2016) HPIP promotes gastric cancer cell proliferation through activation of cap-dependent translation. Oncol Rep 36:3664-3672. https://doi.org/10.3892/or.2016.5157

Coşkun G, Özgür H (2011) Apoptoz ve Nekrozun Moleküler Mekanizması. Çukurova Üniversitesi Tıp Fakültesi Arşiv Kaynak Tarama Dergisi 20:145-158.

Deep G, Raina K, Singh RP, Oberlies NH, Kroll DJ, Agarwal R (2008) Isosilibinin inhibits advanced human prostate cancer growth in athymic nude mice: comparison with silymarin and silibinin. Int J Can 123:2750-2758. https://doi.org/10.1002/ijc.23879

Dinçel GÇ, Kul O (2016) Patolojik Apoptozis ve Tanı Yöntemleri. Gümüşhane University Journal of Health Sciences 5:86-108.

Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495-516. https://doi.org/10.1080/01926230701320337

Eo HJ, Park GH, Song HM, Lee JW, Kim MK, Lee MH, Lee JR, Koo JS, Jeong JB (2015) Silymarin induces cyclin D1 proteasomal degradation via its phosphorylation of threonine-286 in human colorectal cancer cells. Int Immunopharmacol 24:1-6. https://doi.org/ 10.1016/j.intimp.2014.11.009

Eröz R, Karataş A, Alkoç OA, Baltaci D, Oktay M, Çolakoğlu S (2012) Apoptozis Hakkında Bilinenler (Literatür Taraması). Düzce Tıp Dergisi 14:87-101.

Fan L, Ma Y, Liu Y, Zheng D, Huang G (2014) Silymarin induces cell cycle arrest and apoptosis in ovarian cancer cells. Eur J Pharmacol 743:79-88. https://doi.org/10.1016/j.ejphar.2014.09.019

Fujise Y, Okano J, Nagahara T, Abe R, Imamoto R, Murawaki Y (2012) Preventive effect of caffeine and curcumin on hepato-carcinogenesis in diethylnitrosamine-induced rats. Int J Oncol 40:1779-1788. https://doi.org/10.3892/ijo.2012.1343

Gopalakrishnan R, Sundaram J, Sattu K, Pandi A, Thiruvengadam D (2013) Dietary supplementation of silymarin is associated with decreased cell proliferation, increased apoptosis, and activation of detoxification system in hepatocellular carcinoma. Mol Cell Biochem 377:163-176. https://doi.org/10.1007/s11010-013-1582-1

Gupta P, Bansal MP, Koul A (2013) Evaluating the effect of lycopene from Lycopersicum esculentum on apoptosis during NDEA induced hepatocarcinogenesis. Biochem Biophys Res Commun 434:479-485. https://doi.org/10.1016/j.bbrc.2013.03.099

Imamoto R, Okano JI, Sawada S, Fujise Y, Abe R, Murawaki Y (2014) Null anticarcinogenic effect of silymarin on diethylnitrosamine-induced hepatocarcinogenesis in rats. Exp Ther Med 7:31-38. https://doi.org/10.3892/etm.2013.1391

Jin Z, El-Deiry WS (2005) Overview of cell death signaling pathways. Cancer Biol Ther 4:139-163. https://doi.org/10.4161/cbt.4.2.1508

Kaya C, Çalişkan Y, Yönden Z (2012) Apoptozis. Mustafa Kemal Üniversitesi Tıp Dergisi 3: 26-37.

Man S, Li J, Fan W, Chai H, Liu Z, Gao W (2015) Inhibition of pulmonary adenoma in diethylnitrosamine-induced rats by Rhizoma paridis saponins. J Steroid Biochem Mol Biol 154:62-67. https://doi.org/10.1016/j.jsbmb.2015.07.004

Mansour MA, Aljoufi MA, Al-Hosaini K, Al-Rikabi AC, Nagi MN (2014) Possible role of selective, irreversible, proteasome inhibitor (carfilzomib) in the treatment of rat hepatocellular carcinoma. Chem Biol Interact 215:17-24. https://doi.org/10.1016/j.cbi.2014.03.001

Matsuda M, Nakamoto Y, Suzuki S, Kurata T, Kaneko S (2005) Interferon-γ-mediated hepatocarcinogenesis in mice treated with diethylnitrosamine. Lab Invest 85:655-663. https://doi.org/10.1038/labinvest.3700257

Medhat A, Mansour S, El-Sonbaty S, Kandil E, Mahmoud M (2017) Evaluation of the antitumor activity of platinum nanoparticles in the treatment of hepatocellular carcinoma induced in rats. Tumour Biol 39:1-10. https://doi.org/10.1177/1010428317717259

Merhan O, Özcan A, Atakişi E, Öğün M, Kükürt A (2016) The Effect of β-carotene on Acute Phase Response in Diethylnitrosamine Given Rabbits. Kafkas Univ Vet Fak Derg 22:533-537. https://doi.org/10.9775/kvfd.2016.14995

Patial V, S M, Sharma S, Pratap K, Singh D, Padwad YS (2015) Synergistic effect of curcumin and piperine in suppression of DENA-induced hepatocellular carcinoma in rats. Environ Toxicol Pharmacol 40:445-452. https://doi.org/10.1016/j.etap.2015.07.012

Raghunandhakumar S, Paramasivam A, Senthilraja S, Naveenkumar C, Asokkumar S, Binuclara J, Jagan S,

Anandakumar P, Devaki T (2013) Thymoquinone inhibits cell proliferation through regulation of G1/S phase cell cycle transition in N-nitrosodiethylamine-induced experimental rat hepatocellular carcinoma. Toxicol Lett 223:60-72. https://doi.org/10.1016/j.toxlet.2013.08.018

Ramakrishnan G, Jagan S, Kamaraj S, Anandakumar P, Devaki T (2009) Silymarin attenuated mast cell recruitment thereby decreased the expressions of matrix metalloproteinases-2 and 9 in rat liver carcinogenesis. Invest New Drugs 27:233-240. https://doi.org/10.1007/s10637-008-9163-y

Shirakami Y, Gottesman ME, Blaner WS (2012) Diethylnitrosamine-induced hepatocarcinogenesis is suppressed in lecithin:retinol acyltransferase-deficient mice primarily through retinoid actions immediately after carcinogen administration. Carcinogenesis 33:268-274. https://doi.org/10.1093/carcin/bgr275

Shizuma T, Ishiwata K, Nagano M, Mori H, Fukuyama N (2011) Protective effects of fermented rice vinegar sediment (Kurozu moromimatsu) in a diethylnitrosamine-induced hepatocellular carcinoma animal model. J Clin Biochem Nutr 49:31-35. https://doi.org/10.3164/jcbn.10-112.

Sivaramakrishnan V, Niranjali Devaraj S (2009) Morin regulates the expression of NF-kappaB-p65, COX-2 and matrix metalloproteinases in diethylnitrosamine induced rat hepatocellular carcinoma. Chem Biol Interact 180:353-359. https://doi.org/10.1016/j.cbi.2009.02.004

Song Y, Jin SJ, Cui LH, Ji XJ, Yang FG (2013) Immunomodulatory effect of Stichopus japonicus acid mucopolysaccharide on experimental hepatocellular carcinoma in rats. Molecules 18:7179-7193. https://doi.org/10.3390/molecules18067179

Srigopalram S, Jayraaj IA, Kaleeswaran B, Balamurugan K, Ranjithkumar M, Kumar TS, Park JI, Nou IS (2014) Ellagic acid normalizes mitochondrial outer membrane permeabilization and attenuates inflammation-mediated cell proliferation in experimental liver cancer. Appl Biochem Biotechnol 173:2254-2266. https://doi.org/10.1007/s12010-014-1031-y

Subramaniyan J, Krishnan G, Balan R, Mgj D, Ramasamy E, Ramalingam S, Veerabathiran R, Thandavamoorthy P, Mani GK, Thiruvengadam D (2014) Carvacrol modulates instability of xenobiotic metabolizing enzymes and downregulates the expressions of PCNA, MMP-2, and MMP-9 during diethylnitrosamine-induced hepatocarcinogenesis in rats. Mol Cell Biochem 395:65-76. https://doi.org/10.1007/s11010-014-2112-5

Taha MM, Abdul AB, Abdullah R, Ibrahim TA, Abdelwahab SI, Mohan S (2010) Potential chemoprevention of diethylnitrosamine-initiated and 2-acetylaminofluorene-promoted hepatocarcinogenesis by zerumbone from the rhizomes of the subtropical ginger (Zingiber zerumbet). Chem Biol Interact 186:295-305. https://doi.org/10.1016/j.cbi.2010.04.029

Thumvijit T, Taya S, Punvittayagul C, Peerapornpisal Y, Wongpoomchai R (2014) Cancer chemopreventive effect of Spirogyra neglecta (Hassall) Kützing on diethylnitrosamine-induced hepatocarcinogenesis in rats. Asian Pac J Cancer Prev 15:1611-1616. https://doi.org/10.7314/APJCP.2014.15.4.1611

Vásquez-Garzón VR, Macias-Pérez JR, Jiménez-Garcia MN, Villegas V, Fattel-Fazenta S, Villa-Treviño S (2013) The chemopreventive capacity of quercetin to induce programmed cell death in hepatocarcinogenesis. Toxicol Pathol 41:857-865. https://doi.org/10.1177/0192623312467522

Wu T, Liu W, Guo W, Zhu X (2016) Silymarin suppressed lung cancer growth in mice via inhibiting myeloid-derived suppressor cells. Biomed Pharmacother 81:460-467. https://doi.org/10.1016/j.biopha.2016.04.039

Wu WY, Kim H, Zhang CL, Meng XL, Wu ZS (2014) Loss of suppressors of cytokine signaling 3 promotes aggressiveness in hepatocellular carcinoma. J Invest Surg 27:197-204. https://doi.org/10.3109/08941939.2013.873098

Xu X, Fan Z, Kang L, Han J, Jiang C, Zheng X, Zhu Z, Jiao H, Lin J, Jiang K, Ding L, Zhang H, Cheng L, Fu H, Song Y, Jiang Y, Liu J, Wang R, Du N, Ye Q (2013a) Hepatitis B virus X protein represses miRNA-148a to enhance tumorigenesis. J Clin Invest 123:630-645. https://doi.org/10.1172/JCI64265

Xu X, Jiang C, Wang S, Tai Y, Wang T, Kang L, Fan Z, Li S, Li L, Fu J, Liu J, Ji Q, Wang X, Wei L, Ye Q (2013b) HPIP is upregulated in liver cancer and promotes hepatoma cell proliferation via activation of G2/M transition. IUBMB Life 65:873-882. https://doi.org/10.1002/iub.1202

Xu XM, Deng JJ, Yuan GJ, Yang F, Guo HT, Xiang M, Ge W, Wu YG (2011) 5-Lipoxygenase contributes to the progression of hepatocellular carcinoma. Mol Med Rep 4:1195-1200. https://doi.org/10.3892/mmr.2011.547

Zhang XL, Yu H, Xiong YY, Ma ST, Zhao L, She SF (2013) Resveratrol Down-Regulates Myosin Light Chain Kinase, Induces Apoptosis and Inhibits Diethylnitrosamine-Induced Liver Tumorigenesis in Rats. Int J Mol Sci 14:1940–1951. https://doi.org/10.3390/ijms14011940

Zhao JA, Peng L, Geng CZ, Liu YP, Wang X, Yang HC, Wang SJ (2014) Preventive effect of hydrazinocurcumin on carcinogenesis of diethylnitrosamine-induced hepatocarcinoma in male SD rats. Asian Pac J Cancer Prev 15:2115-2221. https://doi.org/10.7314/apjcp.2014.15.5.2115