Effect of Diosmin on The Expression of Epithelial-Mesenchymal Transition Signaling Molecules in Ndea-Induced Hepato-Cellular Carcinoma in Experimental Rats

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Abstract

Hepatocellular carcinoma (HCC) is a primary liver cancer, distinct from other cancers originating in other organs. Previous study demonstrated that diosmin exhibits anticancer effects by influencing the expression of apoptotic signaling molecules in NDEA-induced hepatocellular carcinoma in rats. However, its impact on the epithelial-mesenchymal transition (EMT) signaling pathway, crucial in liver cancer progression, remains unknown. The research aimed to investigate diosmin’s effects on EMT signaling molecule expression in NDEA-induced hepatocellular carcinoma in rats. In this experiment, adult male albino rats were categorized into three groups: a control group, NDEA-induced hepatocellular carcinogenic rats and rats with HCC treated with diosmin orally for 28 days. Liver function markers (AST and ALT) were done by biochemical analysis while mRNA expression analysis of EMT-signaling molecules (E-cadherin and vimentin) were analyzed by Real Time-RT-PCR analysis. One-Way-ANOVA was used for the statistical analysis and significance was considered at p<0.05. Diosmin treatment resulted in a significant decrease in liver function markers compared to the control group (p<0.05). Moreover, diosmin administration led to a notable reduction in mRNA levels of EMT signaling molecules, specifically E-cadherin and vimentin, indicating its potential chemopreventive role against liver cancer. Findings of the present study concludes that diosmin, an alkaloid, may merge as a promising candidate for hepatocellular carcinoma treatment based on its demonstrated efficacy in this experimental model.

Keywords: Novel method, Hepatocellular carcinoma, EMT signaling, diosmin, wistar rats, liver function, innovative technique.

Introduction

Cancer remains one of the most challenging problems of biomedical research in which hepatocellular carcinoma (HCC), a primary liver cancer ranks 6th most diagnosed malignancy [1] and one million death worldwide in a year [2, 3]. The research and therapeutic development strategies for cancers and cancer related diseases are largely based upon a gene mutation model. The complex nature of HCC, dependent on disease staging, location and size of tumor presence. HCC needs a better understanding about its regulatory mechanism [4]. Advances in the understanding of the molecular pathogenesis of HCC have led to identification of critical driver mutations; however, the most prevalent of these are not yet druggable targets [5]. Hence mutations in oncogenes and tumor suppressor genes are the main pathogenic mechanisms underlying hepatocarcinogenesis as they are responsible for disrupting cell signaling pathways. In HCC, the major pathways involved in the oncogenic process are WNT/b-catenin, Hedgehog, hepatocyte growth factor/c-MET, vascular endothelial growth factor (VEGF), mitogen-activated protein kinase (MAPK)/ERK.
(or Ras-Raf-MEK-ERK) and PI3K/AKT/mTOR [6,7]. Among all, the PI3K/AKT/mTOR pathway is particularly interesting because it is constitutively activated in a significant proportion of patients with HCC, and it is associated with a more aggressive tumor progression and shorter survival. The mTOR signaling pathway is frequently deregulated in cancer and metabolic diseases [8,9]. This molecular mechanism is believed to be one of the most attractive targets for the development of mTOR inhibitors which potentially reworks the etiology and pathogenesis of HCC [10,11].

Medicinal plants and plants derived compounds are becoming major targets as therapeutic agents against various deadly disorders, mainly attributed to its least side effects [12-14]. Diosmin is a Plant-derived dietary polyphenolic compound, such as natural flavonoid usually extracted from citrus plants with cancer cell-specific pro-apoptotic activity [15], venoprotective effect, antioxidant, anti-inflammatory and anti-apoptotic activities and chemopreventive potential are thought to be promising anti-cancer agents [16]. Several studies also reported that Diosmin has a beneficial effect in many pathological conditions such as hyperlipidaemia, diabetes mellitus [17] and peptic ulcer [18].

In this study, we have given experimental evidence on the potential role of diosmin that control EMT-Mediated aggravation of hepatocellular carcinoma using NDEA-induced cancer in in vivo model.

**Materials and Methods**

**Animals**

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics Committee (BRULAC/SDCH/SIMATS/IAEC/02-2019/016). Healthy male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180–210 g (150–180 days old) were used in this study. Animals were obtained and maintained in clean polypropylene cages under specific humidity (65 ± 5%) and temperature (27 ± 2°C) with constant 12 h light and 12 h dark schedule at Biomedical Research Unit and Lab Animal Center (BRULAC), Saveetha Dental College & Hospitals, Saveetha Institute of Medical & Technical Sciences, Chennai – 600 077. They were fed with standard rat pelleted diet (Lipton India, Mumbai, India), and clean drinking water was made available ad libitum.

**Experimental Design**

Healthy adult male albino rats were divided into four groups consisting of six animals each. In the present study, diosmin dose (200 mg/kg body weight) was selected based on the study from our laboratory. Group I—Normal control (vehicle treated; DMSO: 1 ml/ kg body weight). Group II—Hepatocellular carcinogenic induced rats (0.01% NDEA orally for 16 weeks). Group III—Cancer-bearing rats were treated with diosmin (200 mg/kg/ body weight/day) orally for 30 days. Group IV—control rats treated with diosmin (200 mg/kg/body weight/day) alone for 30 days. At the end of the experimental period, animals were subjected to ether anesthesia; blood was collected from retro orbital plexus and serum was separated by centrifugation. Animals were sacrificed by cervical decapitation and liver tissues from control and treated animals were excised, washed in ice-cold saline, and blotted to dryness. A 10% homogenate of the tissue was prepared in 0.1 M Tris–HCl buffer (pH 7.4), centrifuged and the clear supernatant was used for further analysis.

**Assessment of Liver Function Markers**

Liver function markers such as alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (AST) kits procured from Spinreact, Spain. Results for same were expressed as U/L [19].

**Real-Time RT-PCR Analysis of mRNA Expression**

The real-time RT-PCR analysis was performed using gene specific primers. cDNA was synthesized from total RNA isolated from
tissues using the first strand synthesis kit (Qiagen, Germany) [20]. The protocol to be used for real-time PCR is as follows: The PCR mixture consists of 5 μl of cDNA sample (1:10 dilution), 300 nM of each primer and 10 μl of master mix for SYBR green I (Eurogentec, Belgium) in a final volume of 20 μl. Amplification was carried out in the MX3000P Multiplex quantitative PCR system with initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 15 seconds annealing at 60°C for 1 min and extension at 72°C for 30 seconds. Amplification of the internal control (β-actin) was performed simultaneously in separate tubes.

All reactions were performed in triplicate along with no template control (NTC) and results were analyzed using MX3000P Multiplex quantitative PCR system software (Stratagene).

Dissociation curve analysis was performed after each reaction to ensure the amplification of a single product. The relative number of mRNAs was calculated using the comparative CT method [20].

**Statistical Analysis**

Data were presented as the mean ± SEM of One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison method was used to compare the means of different groups using SPSS 12.5 student’s versions. p<0.05 was considerable statistically significant in all cases.

**Results**

**Assessment of Liver Function Markers**

Liver function markers ALT, ALT and ALP were significantly raised in NDEA-induced animals compared to control (Figure.1 & 2). Oral administration of diosmin reduced the same significantly (p < 0.05).

X axis represents experimental groups, and the Y axis represents the activity. Each bar represents Mean ± SEM of 3 independent observations. P value ≤ 0.05 was statistically significant. a-compared with control; b-compared with NDEA-Induced rats; c-compared with diosmin.

![Figure 1. Effects of Diosmin on Liver Function Markers in NDEA-Induced Hepatocellular Carcinoma](image-url)
Figure 2. Effects of Diosmin on Liver Function Markers in NDEA-Induced Hepatocellular Carcinoma

Effect of Diosmin on E-Cadherin and Vimentin mRNA Expression in HCC-Induced Animals

In the present study, E-cadherin and vimentin mRNA levels were significantly decreased (p<0.05) in HCC-induced animals compared to control (Figure 7). However, diosmin treatment significantly (p < 0.05) altered the levels of E-cadherin and vimentin and this study shows that diosmin controls EMT signaling in hepatocytes.

Figure 3. Effects of Diosmin on E-cadherin mRNA Expression in NDEA-Induced Hepatocellular Carcinoma

The X axis in Figure 3 indicates different groups, and the Y axis indicates fold change over control. mRNA expression was done by Real Time RT-PCR using rat gene specific primers. Each bar represents Mean ± SEM of 3 independent observations. P value ≤ 0.05 was statistically significant. a-compared with control;b-compared with NDEA-Induced rats.
Figure 4. Effects of Diosmin on Vimentin mRNA Expression in NDEA-Induced Hepatocellular Carcinoma

The X axis in Figure 4 indicates different groups and the Y axis indicates fold change over control. mRNA expression was done by Real Time RT-PCR using rat gene specific primers. Each bar represents Mean ± SEM of 3 independent observations. P value ≤ 0.05 was statistically significant. a-compared with control;b-compared with NDEA-Induced rats.

Discussion

Hepatocellular carcinoma (HCC) accounts for 85 - 90% of liver malignancies [21]. Nitrosamines, dietary carcinogen have been shown to be implicated in the aetiology of HCC, which comprises a wide class of environmental carcinogens found in foodstuffs such as smoked pickled fish, cheese, nitrite-cured meats, dried milk and alcoholic beverages or tobacco smoke. They contribute to the development of chronic inflammation, oxidative stress and cellular proliferation in response to tissue injury, finally leading to hepatic neoplasia [22]. Hepatocarcinogenesis induced by N-nitrosodiethylamine (NDEA), due to histological and biochemical similarities between rodents and human hepatic lesions, is widely used as an experimental model in antitumor studies. Although many progresses have been achieved to demonstrate a strong relationship between cell signaling induce oxidative stress and tumor on HCC research, it still needs a better understanding of the molecular and regulatory mechanisms involved in HCC [23].

A number of epidemiological studies have indicated that a diet rich in fruits and vegetables is associated with the reduction of cancer risk in humans, suggesting that certain dietary constituents may thus be effective in preventing cancer [24]. These natural agents generally down-regulate signaling pathways which have been activated in malignant cells and block the proliferation of initiated cells with minimal damage to normal cells. Most cancer preventive agents are natural phytochemical which act by preventing enzymes involved in carcinogen activation and proliferation [25].

Diosmin is a well-known flavonoid having a broad spectrum of biological activities, including antioxidant [26], anti-inflammatory and anti-apoptotic activities. Several studies reported that diosmin has a beneficial effect in many pathological conditions such as hyperlipidaemia, diabetes mellitus and peptic ulcer [17, 18]. Our previous study [26] also presents that the oral administration of diosmin against N-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis in adult male rats alters the
levels of AFP, LPO, antioxidant enzymes, pro- and anti-apoptotic proteins as well as caspase-3 and -9 proteins.

Moreover, the molecular mechanism of diosmin against the HCC was still unclear. Hence this study is carried out to investigate the molecular mechanism of Diosmin administration in N-nitrosodiethylamine-induced liver cancer.

The generation of oxygen metabolites such as superoxide (\(O_2^-\)), hydrogen peroxide (H2O2) and hydroxyl radical (\(OH^-\)) is believed to be the main cause in the pathogenesis of liver injury. Increased generation of free radicals results in the loss of membrane integrity and function via lipid peroxidation [27]. We assayed some routine clinical chemistry markers to assess the protective influence of diosmin on the liver. As was expected, exposure of rats to NDEA for 16 days caused liver damage, which was evidenced by a rise in the activity of plasma transaminases (ALT and AST), alkaline phosphatase (ALP) along with the rise in peroxyl and hydroxyl radicals. Treatment with diosmin notably reduces the ALT and ALP and thus prevents liver injury. Diosmin inhibits oxidation of low-density lipoproteins in vitro, hence oxidative stress makers in group III-diosmin treated animals significantly reduced near normal (p < 0.05). Diosmin administration to rats showed depression in these liver injury marker enzymes, thus suggesting its cell membrane stabilizing property and hepatoprotective efficacy [28, 29].

Gene Expression levels of E-cadherin and vimentin were studied by Real-Time Quantitative PCR. In NDEA-induced rats, E-cadherin and vimentin mRNA levels were significantly (p<0.05) decreased. In contrast, diosmin treatment upon their levels tends to become normal, indicating diosmin potential to control HCC.

The mechanism of how flavonoids inhibit cancer has also been extensively studied. Diosmin are thought to be ATP analogs capable of blocking the function of various kinases, are actually known to inhibit CDK2 and CDK4, both of which control the function of the anticancer protein retinoblastoma protein [30].

**Conclusion**

In conclusion, our study suggests that diosmin may play a protective effect N-nitrosodiethylamine-induced liver cancer, which could be due to its antioxidant activities that scavenge the free radicals and by protecting liver enzymes. Diosmin targeting on EMT signaling pathway promise provides biological evidence supporting the usefulness of diosmin to protect liver in rats by up regulate e-cadherin and vimentin. Further studies on the effects of diosmin on further downstream signaling molecules to rule out the exact mechanisms to find out whether diosmin can inhibit metastasis on human cell line model are warranted in order develop this natural compound towards clinical trials.

**Conflict of Interest**

The authors hereby declare that there is no conflict of interest in this study.

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**Author Contribution**

1. MB Sai Sreekar Reddy - contributed to designing the study, execution of the project, statistical analysis, manuscript drafting.
2. Dr. Selvaraj - contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.
3. Dr.V. Vishnupriya - contributed in study design, guiding the research work, manuscript correction.
4. Dr. Gayathri R - study design, statistical analysis, manuscript proofreading and correction.
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References


