Modulation of EphA7 and pEphA7 Protein Expression: Potential Biomarkers for Early Detection of Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the leading drivers of cancer-related mortality in the world. As a result, researchers are constantly looking for ways to optimize the screening and diagnosis of the said malignancy. Objective: To establish the mice model of hepatocellular carcinoma with the administration of a suitable dose of diethylnitrosamine (DEN) and examine the utility of EphA7 and pEphA7 as ideal diagnostic markers in HCC. Methods: Swiss Albino (BALB/c) mice of around 10-12 weeks old were exposed to a known hepatocarcinogen-diethylnitrosamine at a dose of 20 mg/kg body weight at weekly intervals for a period of 4, 8, 12, & 16 weeks. Blood was collected from mice of different experimental groups, and age-matched control and serum were separated from whole blood samples. The liver homogenate was prepared after completion of treatment, and the resulting supernatant was used for enzyme assays. A range of liver biomarker enzyme assays such as Gamma-glutamyl transpeptidase (GGT), Acetylcholine esterase (AChE), GPx activity and GSH level, Heme oxygenase-1 (HO-1), GPC3 and alpha-fetoprotein (AFP) level along with the expression of Caspase-3, EphA7 and pEphA7 were evaluated. Results: An elevation in body weight and relative liver weight across the treatment period (4, 8, 12, 16 weeks) was observed in DEN-treated mice. Significant differences in GGT levels between control and DEN treated mice were noted in the present study (P < 0.005). In the 16th week of the treatment period, a significant difference in AchE level was noted between the treated and control group (P < 0.001). However, there was no statistically significant difference in the levels of SGOT and SGPT levels between the control and DEN treated groups (P > 0.001). Lower GSH and GPx levels were demonstrated in the treated mice as compared to control over all the treatment period. Loss of Caspase-3 expression and significant differences in expression of HO-1 activity in treated vs. non-treated group of mice were observed. Significant differences in EphA7 and pEphA7 protein expression levels were noted in the DEN-treated vs. control groups across all the treatment periods (4 weeks: P < 0.05; 8 weeks: P < 0.05; 12 weeks: P < 0.005; 16 weeks: P < 0.05). Conclusion: The present study indicated that EphA7 and phosphoEphA7 over-expression might contribute to the malignancy transition, invasion development, and metastasis of HCC. As a result, along with the known markers such as AFP and others, EphA7 and pEphA7 could be a very putative biomarkers of HCC, particularly at a very early stage of cancer development.

Keywords: Hepatocellular carcinoma- biomarker- diethylnitrosamine- EphA7

Introduction

With a projected incidence of approximately >1 million cases by 2025, liver cancer continues to be a serious health challenge (Villanueva, 2019). The most prevalent form of liver cancer, hepatocellular carcinoma (HCC), accounts for the majority of all occurrences. The predominant risk considerations for HCC progression include hepatitis B and C virus infection while non-alcoholic steatohepatitis linked with metabolic syndrome or diabetes mellitus has become a more recurring problem in the West (Llovet et al., 2021). The most successful treatment for HCC is radical tumor excision, but the risk of recurrence is still substantial, with most instances recurring in the intrahepatic zone (Minagawa et al., 2003). Regrettably, the majority of HCC patients experience relapses within two years of surgery (Imammura et al., 2003). Efforts have been undertaken to uncover factors that influence patient survival, and some researchers have focused on the function of cancer cell viability, which is thought to be attributable to over-expression of several cytoprotective proteins (Schoemaker et al., 2002).

The pathophysiological underpinnings of HCC are yet unknown. As a result, appropriate experimental HCC models with or without cirrhosis traits that are comparable to HCC must be identified. Preclinical studies in vivo...
are becoming increasingly important in the research of 
HCC (De Minicis et al., 2013). HCC genesis is mostly studied using genetic, transgenic, or conditional 
knock-out experimental models (Feng et al., 2011). 
Diethylnitrosamine (DEN) is widely used as a carcinogen 
in animal models to induce hepatocarcinogenesis and 
and its mechanism of action is well-documented (Hadem et 
al., 2014; Hamza et al., 2021). DEN is a pro-carcinogen 
and it require metabolic activation by Cytochrome P450 
2E1 (CYP2E1) a part of CYP 450 enzyme families 
(Farhat et al., 2019). The metabolically active compound 
diureasate ethyl-adduct leading to hepatocellular cancer (Helms et al., 2021). a representative chemical 
carcinogen with the ability to develop tumors in multiple 
organs, including the liver, skin, gastrointestinal tract, 
liver, and respiratory system, is known to promote HCC 
in rodents. Cell cycle regulatory proteins were found to be 
crucial in cancer start and also progression by DEN. The 
use of DEN in rats has become a popular experimental 
paradigm for investigations focused on comprehending 
the pathogenetic changes implicated in the development 
of liver cancer, which is one of the most prevalent cancer 
in humans (Tolba et al., 2015). A single injection dose of 
DEN ranging from 1.25 to 25 mg/kg body weight in two- 
week-old mice, when hepatocytes are in a proliferating 
state, results in the emergence of HCC in a time span of 
approximately 8–12 months (Hacker et al., 1991). The 
proximity of gene expression sequences to human HCC 
is a benefit of chemically induced models, particularly 
the DEN-induced HCC mouse model (Kamino et al., 
2011). Proliferation and chromosomal instabilities are 
all elevated in DEN-induced tumors, as are low levels of 
β-catenin mutation and apoptotic rate in human HCC with 
a sub-optimal prognosis (Wang et al., 2012).

To enhance the prognosis of the said carcinoma, 
researchers are constantly striving to boost screening, 
diagnostic, and treatment strategies. Current serum 
biomarkers demonstrate low sensitivity and diverse 
specificity despite multiple cut-off values. Identifying 
visible biomarkers for monitoring and early HCC diagnosis 
is still lacking. HCC biomarkers employed for prognostic 
or predictive objectives, on the other hand, may play a 
more significant role in clinical practice in the near future. 
The present study attempts to establish the mice model 
of hepatocellular carcinoma with the administration of 
a suitable dose of diethylnitrosamine (DEN) and examine the 
utility of EphA7 and pEphA7 (formally known as MDK1 OR EHK3) used as an ideal diagnostic marker for 
early detection of HCC.

**Materials and Methods**

Swiss Albino (BALB/c) mice around 10-12 weeks 
old were procured from the Pasteur Institute Shillong, 
Meghalaya, India. Animals were bred at the animal 
house by random inbreeding and were kept on a 
basal diet *ad libitum* and housed in plastic cages in a 
temperature-controlled animal room (21 ± 2°C) with a 12 
h light-dark cycle. The Institutional Ethics Committee of 
North-Eastern Hill University, Shillong has approved the 
experimental protocols.

**Carcinogen exposure protocol**

Mice were exposed to a known hepatocarcinogen-
diethylnitrosamine (DEN) for a period of 4, 8, 12 and 
16 weeks. DEN was prepared in Millipore water and 
administered intravenously at a dose of 20 mg/kg body 
weight at the weekly interval, starting with week 0 (start 
of the experiment) and terminated at week 16. Age and 
sex-matched untreated mice were used as controls.

**Serum collection**

Blood was collected from mice of different 
experimental groups, and age-matched control and serum 
were separated from whole blood samples. Blood was 
collected by retro-orbital bleeding from the corner of the 
eyes using sterilized capillary tubes and kept for 1 h at 
4 °C. Then it was centrifuged at 2,000 x g for 30 min at 
4 °C, and serum was collected.

**Preparation of liver homogenate**

The liver homogenate was prepared in after completion 
of treatment, mice were sacrificed under anesthesia and 
the liver was excised and removed quickly, rinsed in 
ice-cold normal saline (0.9% sodium chloride), blotted 
dry and weighed. The tissue 10% w/v pooled from mice 
was homogenized by 30 strokes with a homogenizer in 
ice-cold 0.25 M sucrose. A homogenate was then prepared 
in chilled 0.25 M sucrose solution and centrifuged at 
20,000 x g for 30 min at 4 °C. The resulting supernatant 
was used for enzyme assays. Total protein content was 
estimated by Bradford’s method.

**Serum marker assay**

Liver injury/disease markers like aspartate transaminase 
(AST) and alanine transaminase (ALT) were measured by 
using the kit (Coral Clinical System, Goa, India).

**Liver marker enzyme Assay**

Liver marker enzymes Gamma-glutamyl transpeptidase 
(GGT) and Acetylcholine esterase (AChE) activities assay 
were carried out.

**AChE activity**

Liver marker enzyme AChE activity was determined 
according to the method reported by Ellman (Ellman 
1961).

For the AChE activity assay, 2,800 µl of buffer, 50 
µl of AcSCHI and 100 µl of DTNB was taken in a 3 ml 
cuvette with a light path of 1 cm and mixed thoroughly. 
The reaction was started by adding 50 µl of sample and 
was monitored by reading the absorbance at 412 nm with a 
time interval of 30 sec for 3 min against a blank containing 
all the components except the sample. The increase in 
absorbance min-1 was recorded and the temperature was 
maintained at 30 °C throughout. The specific activity of 
the enzyme was calculated and expressed as U/µg Protein.

**Estimation of Gamma-Glutamyl Transferase**

Added 50 µl serum/liver homogenate, 450 µl 
pro-warmed buffer substrate, incubated at 37 °C for 30 
min, then added 2.5 ml acetic acid to the reaction and read 
the color at 405 nm against a blank prepared by adding
50 μl serum/liver homogenate to 450 μl buffer substrate and 2.5 ml acetic acid (Rosalki et al., 1970).

**Antioxidant enzymes assay**

GPx activity and GSH level were analyzed by following methods reported by Rahman et al., (2006) with some modifications.

**Western Blotting**

Tissue lysate was subjected to SDS-PAGE followed by transfer to nitrocellulose membrane. The membranes were blocked in 5% non-fat skim milk for 1 hour followed by washing and then incubated in EphA7 and pEphA7 primary antibody from sigma (1:1,000) in 5% non-fat skimmed milk overnight at 40 °C. This was followed by incubation in secondary antibody conjugated to alkaline phosphatase and then followed by color development using BCIP/NBT solution.

**Caspase-3 activity**

The assay was performed as per the manufacturer’s instructions. Tissue homogenate was prepared and then lysate was incubated with the substrate DEVD-pNA at 37 °C for 2 hours, after which the optical readings were taken at 400 nm.

**Serum Biomarker AFP and GPC3 estimation**

GPC3 and alpha-fetoprotein levels were measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit and were performed according to the manufacturer’s instructions. In brief, serum was added to 96 well-plates and an antibody cocktail was added. Then the plate was kept for 1h incubation at room temperature. Then washing was done by using wash buffer and TMB substrate was added to each well and was incubated for 10 min in dark and reaction was stopped by using stop solution and absorbance was recorded at 450 nm.

**Results**

The tumor model of DEN was established at a dose of 20 mg/kg body weight, and their change in body weight and relative liver weight was observed (Figure 1). An elevation in body weight and relative liver weight across the treatment period (4, 8, 12 and 16 weeks) was observed in DEN-treated mice. Significant differences in GGT levels between control and DEN-treated mice was noted in the present study (P < 0.005). In the 16th week of the treatment period, a significant difference in AchE level was noted between the treated and control group (P < 0.001) (Figure 1). However, there was no statistically significant difference in the levels of SGOT and SGPT levels between the control and DEN-treated groups (P > 0.001). Loss of Caspase-3 expression and significant differences in expression of HO-1 activity in treated vs. non-treated group of mice were observed. Significant differences in EphA7 and pEphA7 expression levels were noted in the DEN-treated and control groups over the treatment period (4 weeks: P < 0.05; 8 weeks: P < 0.05; 12 weeks: P < 0.005; 16 weeks: P < 0.05). Lower GSH and GPx levels were demonstrated in the treated mice as compared to control over all the treatment period (Figure 2). Higher biomarker protein GPC3 and AFP level in serum of treated mice was demonstrated in the present study upon treatment of DEN (Figure 3). Loss of Caspase-3 expression and significant differences in expression of HO-1 activity in treated vs. non-treated group of mice were observed (Figure 4).

Significant differences in EphA7 and pEphA7 protein levels were noted in the DEN-treated groups vs.
respective control groups across all the treatment periods (Figure 5). The higher protein level of EphA7 was more pronounced at 12 and 16 weeks treated groups compared to their respective controls as shown in the Western blot analysis (Figure 5 A and B). For pEphA7, the higher protein level was observed in all the treated groups vs. respective controls. However, the difference were less pronounced compared to the EphA7 levels in different groups (Figure 5 A and C). Our result corroborates earlier findings of role of EphA7 as a tumor promoter in multiple cancers (Chen et al., 2022; Zhang et al. 2022).

**Discussion**

Diethylnitrosamine (DEN) is a potent hepatocarcinogen that has been shown to cause cancer in mice (Sreepriya et al., 2005). An elevation in body weight across the treatment periods (4, 8, 12 and 16 weeks) was observed in DEN-treated mice which may be due to the elevated metabolic rate of the liver and higher buildup of high-density lipoproteins. The weights of the livers in the DEN-treated group similarly increased. A study found that when mice were given DEN, their liver weight increased nearly two-fold (Kowsalya et al., 2015).

Gamma-glutamyl transpeptidase (GGT) levels
have been suggested to be a potentially useful HCC biomarker. Significant differences in GGT levels between control and DEN-treated mice were noted in the present study (P < 0.005). In healthy individuals, GGT levels were noted to be modest; however, the level hikes up in patients with benign or malignant liver carcinoma (Zhao et al., 2013). In the 16th week of the treatment period, a significant difference in AchE level was noted between the treated and control group (P < 0.001). However, there was no statistically significant difference in the levels of SGOT and SGPT levels between the control and DEN-treated groups (P > 0.001). All the increased levels of the said biomarkers indicate the induction of hepatic carcinoma upon treatment with DEN (Tata 2008; Tolba et al., 2015; Kulkarni et al., 2016).

Lower GSH and GPx levels were demonstrated in the treated mice as compared to control over all the treatment period. GSH is involved in the development, proliferation, development, and metastasis of HCC tumors (Traverso et al., 2013). HCC patients reported reduced plasma levels of GSH and GSH-related antioxidant enzymes than healthy controls or normal participants, patients with hepatitis, or liver cirrhotic patients in prior studies (Shimomura et al., 2017). Because of the existence of the HCC tumor, GPx, a phase I antioxidant enzyme, becomes exhausted, which might be a reason for lower level of GPx in the treated group of mice. Reduced systemic GPx3 levels have been found in experimental studies employing mice models to enhance colon tumor development and carcinogenic stimulation (Barrett et al., 2013). Patients with a lower plasma GPx3 level had a decreased 5-year recurrence rate than patients with a greater plasma GPx3 level, according to a study report published by Qi et al., (2014).

Loss of Caspase-3 expression was demonstrated by the treated mice over time. It is believed that the reduction of Caspase-3 expression may represent a crucial role in HCC since Caspase-3 is the effector caspase in the apoptotic cell death processes (Sun et al., 2000). Heme oxygenase-1 (HO-1) is linked to tumor metastasis and is known to perform a key role in antioxidative and antiapoptotic processes (Jozkowicz et al., 2007). The present study showed significant differences in the expression of HO-1 activity in treated vs. non-treated groups of mice. Positive HO-1 expression was discovered in 44.8% of the cases, and it was most commonly identified in patients with locally progressed histology of the said cancer (Park et al., 2019).

Higher levels of biomarker proteins- GPC3 and AFP level in serum of treated mice was demonstrated in the present study upon treatment of DEN. GPC3 expression was found in 60% of five atypical hepatocellular adenomas studied by Wang et al., (2006), implying that GPC3 could be beneficial in conditions of malignant development. GPC3 is not found in the hepatocytes of healthy
individuals, however, it can be found in roughly 50% of HCC patients and 33% of HCC patients who are AFP-negative (Nakatsura et al., 2003). Based on the reported evidences, it may be stated that GPC3 could be used as a biomarker in the early stages of hepatocarcinogenesis. In addition, HCC, embryonic carcinomas, and gastric and lung cancer are all reported to have high levels of AFP (Grizzi et al., 2007). About 50% of HCC patients produce AFP, which is utilized in the differentiated detection and follow-up in HCC patients (Zhang et al., 2014).

Tyrosine kinases, which are important signal transduction pathway mediators, are linked to processes such as cellular proliferation, apoptosis, and cancer (Dodelet et al., 2000). Eph receptor tyrosine kinases (Ephs) and their membrane-appended ephrin ligands, ephrins create a cellular system linked to cellular attachment or migration, angiogenesis, and tumor vasculature in a variety of human carcinoma cells (Nakamoto et al., 2002; Clevers et al., 2006). Many Ephs and their ligands, ephrins, are expressed in tumor tissues during maturity, and they are hypothesized to play a role in tumor invasion and metastasis (Brantley-Sieders et al., 2004; Héroult et al., 2006). EphA3 is a member of the Eph/ephrin tyrosine kinase system, which is involved in vasculature formation and could impact tumor angiogenesis (Davies et al., 2005; Xi et al., 2012). Cellular proliferation and survival, reduction of cell adherence to fibronectin, cell migration, and anti-apoptosis are all impacted by alterations in EphA receptors. Abnormal EphA7 modulation and genetic changes have been linked to the genesis and advancement of various malignancies (Bardelli et al., 2003; Hafner et al., 2004; Lee et al., 2006; Bonifaci et al., 2010). Significant differences were also noted in the treated and control groups over the treatment period (4 weeks: P < 0.05; 8 weeks: P < 0.05; 12 weeks: P < 0.005; 16 weeks: P < 0.05). Similar results were reported in a study where the protein expression level of EphA7 and pEphA7 protein in HCC was higher than in normal liver tissues (P < 0.05), as evidenced by Western blot analysis (Figure 5). Zhang et al., (2010) have reported the same evidence as histopathological segmentation, tumor thrombi in the portal vein, lymph node metastases, and elevated AFP levels were linked to EphA7 protein expression (P < 0.05). The biological activity of EphA7 and pEphA7 over-expression might contribute to the malignancy transition, invasion development, and metastasis of HCC. Obviously, reliable biomarkers for HCC would be highly desirable. It could help clinicians detect the disease at a very early stage and enable them to select viable choices for appropriate therapeutic approaches at a very early stage of carcinogenesis. The present study indicated that EphA7 and phosphoEphA7 over-expression might contribute to the malignancy transition, invasion development, and metastasis of HCC. As a result, along with the known markers such as AFP and others, EphA7 and pEphA7 could be putative biomarkers of HCC, particularly at a very early stage of cancer development.

Author Contribution Statement

Both the authors have contributed equally in the carrying out the experiments, interpretation of data, preparation and presentation of the entire submitted manuscript.

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