

RESEARCH ARTICLE

Tissue Inhibitor of Metalloproteinase 3 as a potential staging biomarker in hepatocellular carcinoma

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Abstract

Objective: To assess the potential role of tissue inhibitor of metalloproteinase 3 as a staging marker of hepatocellular carcinoma.

Method: The experimental study was conducted at Faculty of Pharmacy, Kafrelsheikh University, Egypt, from December 2020 to March 2022 after approval from the Faculty of Pharmacy, Kafrelsheikh University, Egypt, and comprised male albino rats. The subjects were divided into 4 groups. The control group was administered a single intraperitoneal injection of normal saline, while the other groups were generated post-induction of hepatocellular carcinoma. The induction was done by injecting rats intraperitoneally with a single dose of 200mg/kg diethyl nitrosamine, followed by the administration of 0.05% phenobarbital sodium in drinking water daily. Rats were euthanised at 8, 16 and 24 weeks after the injection to obtain three groups related to the 3 stages of hepatocellular carcinoma. Serum was used for measuring the alpha protein level. Liver homogenates were used for the assessment of the hepatic tissue inhibitor of metalloproteinase 3 expression, B-cell lymphoma 2-associated X protein expression, and the hepatic content of matrix metalloproteinase -9 and cyclin D1. Data was analysed using Graph Prism 8.

Results: Of the 24 rats with weight range 120-130g, 6(25%) were in each of the 4 groups. The relative protein and messenger ribonucleic acid tissue inhibitor of metalloproteinase 3 expressions were significantly decreased in the intervention groups compared to the control group, with more decline as the hepatocellular carcinoma stage increased. The matrix metalloproteinase -9 and cyclin D1 concentrations and the relative hepatic protein Ki67 expression were significantly raised in the intervention groups compared to the control group ($p < 0.05$). The relative expression of hepatic B-cell lymphoma 2-associated X protein was markedly decreased in the intervention groups compared to the control group ($p < 0.05$).

Conclusion: Tissue inhibitor of metalloproteinase 3 might be a promising diagnostic and staging biomarker in hepatocellular carcinoma.

Keywords: Carcinoma, Hepatocellular, Neoplasms, Fetoproteins, Metalloproteinase, Cyclin, Antigen, Phenobarbital, Nitrosamines. **DOI:** 10.47391/JPMA.EGY-54-7

Introduction

Hepatocellular carcinoma (HCC) is the most frequent tumour of liver malignancy and it is the leading cause of cancer-associated deaths worldwide.¹ Staging of HCC patients is an important issue in oncology practice², as it is required for predicting HCC prognosis, facilitating the selection of the most appropriate cancer therapy, determining overall survival (OS), and allowing comparison of research results.³ Numerous staging systems help medical experts in the management of cancer.⁴ Until now, the expected performance of the present cancer classification systems has not been ideal as they have numerous limitations. Different novel serological and histological biomarkers are still under examination with promising results, but they require further evaluation.⁵

Tissue inhibitors of metalloproteinases (TIMPs) are ubiquitously expressed proteins that can reversibly suppress the action of the zinc protease enzymes, including a disintegrant and metalloproteases (ADAMs) and matrix metalloproteinases (MMPs). Tissue inhibitor of metalloproteinase-3 (TIMP-3), an endogenous inhibitor of MMPs, regulates several processes, such as matrix regulation, angiogenesis, apoptosis and inflammation⁶. The expression of TIMP-3 is downregulated in different types of cancers, including liver cancer.⁷ The role of TIMP-3 as a staging biomarker in HCC has not been studied. The current study was planned to fill the gap by assessing the potential role of TIMP-3 as a HCC staging marker.

Materials and Methods

The experimental study was conducted at Faculty of Pharmacy, Kafrelsheikh University, Egypt, from December 2020 to March 2022 after approval from the ethics review committee of the Faculty of Pharmacy, Kafrelsheikh University, Egypt, male albino rats were acquired. All animals were kept in sanitary conditions with a 12-hour

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light/dark cycle, mean temperature $25\pm 2^{\circ}\text{C}$, and were provided free access to water and food.

The subjects were divided into 4 groups. The control group was administered a single intraperitoneal (IP) injection of normal saline, while the other groups were generated post-induction of HCC. The induction was done by injecting rats with a single IP dose of 200mg/kg diethyl nitrosamine (DENA) with 99% purity (Toronto Research Chemicals, Toronto, Canada) which was freshly prepared and dissolved in normal saline. This was followed 14 days later by the administration of 0.05% phenobarbital sodium (PB) with 98% purity (Alpha-Chemika, Maharashtra, India) in drinking water daily⁸. Rats were euthanised at 8, 16 and 24 weeks after the DENA injection to obtain groups 2, 3 and 4 related to HCC stage 1, 2 and 3, respectively (Figure 1).

Blood samples were obtained through puncture of the retroorbital venous plexus of the rats under anaesthesia with diethyl ether⁹ followed by centrifugation for 30 min to obtain sera specimens. The collected sera were then frozen at -80°C for further biochemical assessment.

After the animals were euthanised, their whole livers were isolated and divided into three parts. The first part, used for histopathological and immunohistochemical (IHC) investigation, was fixed in phosphate-buffered formalin (PBF; 10%; pH 7.2). The second portion was kept in ribonucleic acid-based RNA lysis solution and stored at -80°C for real-time polymerase chain reaction (RT-PCR). The last portion was used for obtaining liver homogenate using radioimmunoprecipitation assay (RIPA) buffer with inhibitors of protease and phosphatase. Liver homogenate samples were then stored at -80°C for further analyses.

Quantitative RT-PCR (qRT-PCR) technique was used to detect the hepatic B-cell lymphoma 2-associated X protein (Bax) and TIMP-3 messenger RNA (mRNA) expressions. Quantification of the RNA amount was performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (Fermentas, United States). The yield of the obtained total RNA was assessed using a spectrophotometer at 260nm where the absorbance unit (A260) equalled 40 μg of single standard RNA/ml. QuantiTect reverse transcription kit (Qiagen, USA) was used for reverse transcription of 1 μg of total RNA into single-stranded complementary deoxyribonucleic acid (cDNA). The housekeeping gene was β -actin. Forward and reverse primers' sequences were noted (Table 1).

The PCR protocol entailed incubation 5 minutes at 95°C , followed by another incubation at 94°C for 20 seconds, and at 60°C for 1 minute (40 cycles). The comparative Ct method ($\Delta\Delta\text{CT}$) was used to calculate the relative expression levels.

The expression of TIMP-3 protein in hepatic homogenates from different studied rat groups was assessed using western blot analysis for which 5 mg of liver sample was homogenised using RIPA buffer pre-treated with a protease and phosphatase inhibitor cocktail followed by centrifugation. The protein concentration was detected in the collected supernatant based on the Bradford assay.¹⁰ Approximately 30 μg of total protein concentration was isolated by electrophoresis on a 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose membranes. Blots were then blocked in 3% bovine serum albumin and incubated in the diluted TIMP-3 primary antibody for 12 hr at 4°C (ABclonal company, USA; dilution 1:1000, Catalogue No. A11986). The blots were incubated in horseradish peroxidase-conjugated polyclonal secondary antibody (Abcam Inc, USA, Catalogue No. ab6721) at room temperature for 1 hr. TIMP-3 protein was then detected with enhanced chemiluminescence (ECL) detection reagents.

Serum specimens were used for the determination of alpha-fetoprotein (AFP) concentration using the AFP enzyme-linked immunosorbent assay (ELISA) Kit (My BioSource, USA, Catalogue No. MBS267612). On the other hand, liver homogenate samples were used for the ELISA assay of hepatic concentrations of cyclin D1 and matrix metalloproteinase 9 (MMP-9) (Catalogue No. LSF11068, and MBS722532, respectively, My BioSource, USA).

Fixed hepatic tissues in 10% buffered formalin were dehydrated by dipping them in serial ascending dilutions of ethyl alcohol. Liver tissues were then embedded into paraffin blocks, which were sliced into 5 μm -thick sections. Hepatic sectors were then stained using haematoxylin and eosin (H&E) stain. Histopathological alterations were investigated using a light microscope.

IHC staining of Ki67 was performed based on the Saber et al. protocol.¹¹ Liver sections were dewaxed and then incubated with Ki67 polyclonal rabbit antibodies (1:200 dilution, Abcam Inc., USA). Sections were then rinsed with phosphate buffer saline (PBS) and incubated with horseradish peroxidase secondary antibody at room temperature for 30 min. After that, 3,3'-Diaminobenzidine (DAB) kit was used for slide visualisation. Image J software (NIH, USA) was used to evaluate the intensity of staining, and was expressed as a percentage of positive cells/1000 cells in about 8 fields using high power.

Data was analysed using Graph Prism version 8. Data was expressed as mean \pm standard deviation (SD) and as frequencies and percentages, as appropriate. The experimental findings were assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc

test. Ki67 data was interpreted using Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Of the 24 rats with weight range 120-130g, 6(25%) were in each of the 4 groups. Protein and mRNA TIMP-3 expressions were markedly low in the DENA-treated groups compared to the control group ($p < 0.05$). HCC stage 2 group showed a marked decrease in TIMP-3 hepatic expression compared to the HCC stage 1, and HCC stage 3 group showed a more marked decline in TIMP-3 hepatic expression compared to HCC stage 2 group (Figure 2A-B).

The hepatic concentration of MMP-9 was markedly elevated in the DENA-treated groups compared to the control group ($p < 0.05$). HCC stages 2 and 3 groups showed a marked rise in TIMP-3 hepatic expression in comparison with HCC stage 1 group (Figure 2C).

The serum AFP level was markedly increased in the DENA-treated groups compared to the control group ($p < 0.05$). It was significantly increased in HCC stage 3 compared to stage 2, which displayed a marked elevation compared to stage 1 (Table 2).

The relative hepatic Bax mRNA expression was markedly decreased and the hepatic cyclin D1 was significantly increased in the DENA-treated groups compared to the control group ($p < 0.05$). The HCC stage 2 group displayed

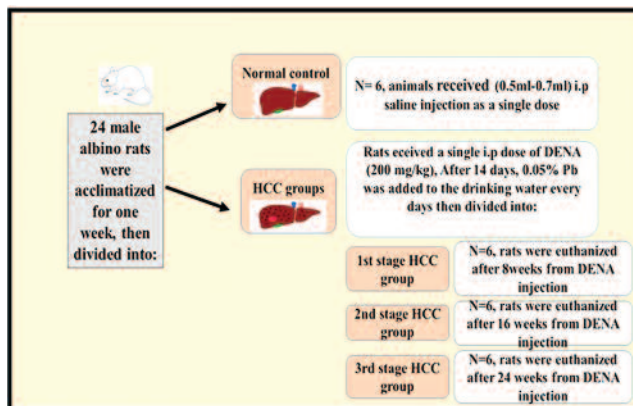


Figure 1: Schematic representation of the experimental design.

DENA: Diethyl nitrosamine, Pb: P; HCC: Hepatocellular carcinoma.

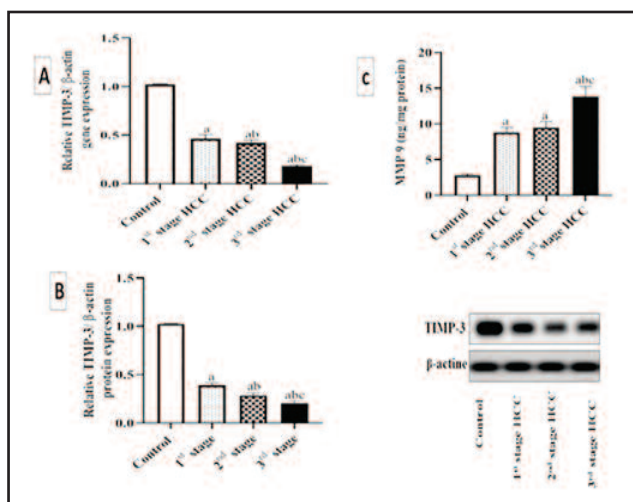


Figure 2: Changes in the relative expression of TIMP-3 mRNA (A) and protein (B), and hepatic content of MMP-9 in liver homogenate among different HCC stages. TIMP-3: Tissue inhibitor matrix metalloproteinase-3, mRNA: Messenger ribonucleic acid, MMP-9: Matrix metalloproteinase-9, HCC: Hepatocellular carcinoma. a, b and c were had statistically significant difference compared to the control group.

Table-1: Forward and reverse primer sequences.

Gene	Primer sequence	Reference
Bax	F:5'CGGCGAATTGGAGATGAAGTGG3' R:5'CTAGCAAAGTAGAAGAGGGCAACC3'	28
TIMP-3	F:5'-GCCTTCTGCAACTCCGACATC-3' R:5'-CGTGATCATCTGCCATCATA-3'	29
β-actin	F:5'-GATGGTGGGTATGGGTCAGAAGGAC-3' R:5'-GCTCATTGCCGATAGTGATGACT-3'	30

Bax: B-cell lymphoma 2-associated X protein, TIMP-3: Tissue inhibitor matrix metalloproteinase-3, F: Forward, R: Reverse.

Table-2: The variation in serum alpha fetoprotein (AFP) and hepatic expression of B-cell lymphoma 2-associated X protein (Bax) messenger ribonucleic acid (mRNA) and cyclin D1 among the four studied rat groups.

Group	serum AFP (ng/ml)	Relative Bax/β-actin gene expression	Cyclin D1 (pg/mg protein)
Control	4.550±0.38	1.0±0.05	54.70±1.669
1st stage HCC	10.12±0.99a	0.58±0.03a	142.4±11.04a
2nd stage HCC	11.88±1.01ab	0.49±0.02ab	147.6±9.574a
3rd stage HCC	14.70±1.39abc	0.27±0.03abc	207.6±19.50abc

HCC: Hepatocellular carcinoma.

a, b, and c were considered as the statistic significant difference versus the control group, the 1st stage HCC group, and the 2nd stage HCC group at $p < 0.05$.

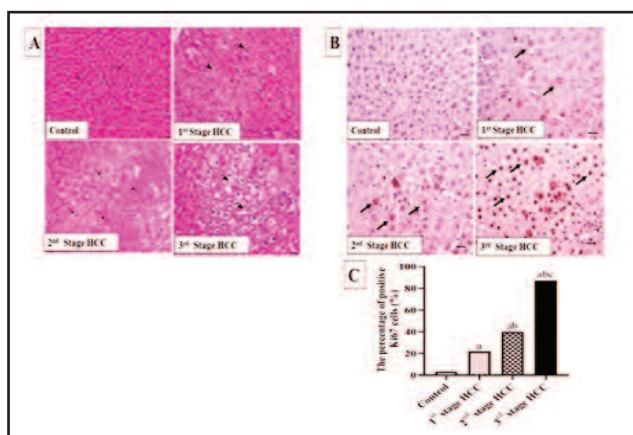


Figure 3: (A) Schematic micrograph of histopathological changes in different stages of hepatocellular carcinoma (HCC). Bar = 50µm, Haematoxylin and eosin (H&E), X200. (B) Microscopic sections from liver specimens immunostained against Ki67 showing the variation in Ki67 expression in different HCC stages. Bar = 50µm, X: 400. (C) Variation in the percentage of hepatic cells showing positive expression of Ki67/1000 cells in different HCC stages.

a marked alteration in Bax and cyclin D1 hepatic expressions compared to HCC stage 1 group, and HCC stage 3 group exhibited a marked Bax and cyclin D1 alteration compared to HCC stage 2 group ($p < 0.05$).

Histopathological examination of liver tissues showed normal hepatocytes (H) in the control group with small uniform nuclei and granulated cytoplasm around the central vein (CV). On the other hand, preneoplastic nodules (N) of clear type were observed in HCC stage 1 group, which showed cytomegaly, and double nuclei in hepatic cells (marked with arrowhead). The HCC stage 2 group revealed preneoplastic foci of a clear type with vacuolar features (marked with black arrows) and a centrilobular area displaying distinct degeneration and necrosis of hepatic cells (marked with arrowheads). The HCC stage 3 group revealed clear-type preneoplastic nodules (N), which were large in size with nuclear pyknosis (marked with arrowheads), cytoplasmic enlargement and vacuolation (Figure 3A).

The immunostaining of liver tissues against Ki67 displayed a low number of hepatocytes stained by the Ki67 antibody in the control group. The number of positive cells stained with Ki67 (brown colour) significantly increased in the HCC stage 1 group, and increased even more in the HCC stage 2 group, and the most in the HCC stage 3 group (Figure 3B-C).

Discussion

The MMP inhibitor family appears to have anti-tumour properties, such as apoptosis induction and antiangiogenic, as well as anti-proliferative and anti-metastatic properties.¹² It is known that the AFP biomarker loses high sensitivity and prognostic value in the detection of HCC, and using AFP for HCC diagnosis shows less specificity than expected as its level may be high in hepatic cirrhosis and cancers other than HCCs. As such, further studies are needed to search for a more ideal tumour biomarker for HCC diagnosis and prognosis.¹³

Several studies have revealed the anti-cancer role of TIMP-3.^{14,15} The current study illustrated that the TIMP-3 expression was downregulated in the DENA-treated HCC groups, with a significant difference between the various stages of HCC. The findings indicate a promising role for TIMP-3 as a staging biomarker in HCC.

Tumour cells grow in an uncontrolled manner, and they can evade the immune system. The balance between cyclin-dependent kinases (CDKs) and their inhibitors controls cell proliferation. One of the important cell cycle progression markers is cyclin D.¹⁶ In several cancer cell lines, the upregulation of TIMP-3 expression inhibits cancer cell growth and promotes apoptosis.¹⁷ The current study

revealed that the downregulation of TIMP-3 expression was associated with increased tumour proliferation and cell cycle markers as the expression of Ki67 and cyclin D1 was markedly increased in the three HCC stage groups in comparison with the control group. Overexpression of TIMP-3 was found to cause a dose-dependent suppression of the increased cell number, but it promoted S-phase entry, which is explained by cells accumulation in the Growth 2/Mitosis (G2/M) phase and the apoptotic-induced cells death.¹⁸ A previous study also revealed that cyclin D1 was overexpressed in TIMP-3^{-/-} cardiomyocytes and downregulated in cells treated with recombinant TIMP-3 (rTIMP-3) in contrast to the wild-type.¹⁶ Another study revealed the inhibitory role of TIMP-3 on cell proliferation as TIMP-3 knockdown in H9c2 cells markedly elevated cyclin D2 and Ki67.¹⁹

Along with reduced cell proliferation, the current study also showed that TIMP-3 downregulation caused a marked decrease in the expression of the apoptotic marker Bax. TIMP-3's role in mediating apoptosis has attracted high attention. It was found that TIMP-3 plays a role in inducing the intrinsic apoptotic pathway.²⁰ Another study revealed that TIMP-3 overexpression caused an increase in the proapoptotic protein expression Bax, and a decrease in the expression of the anti-apoptotic proteins B-cell lymphoma extra large (Bcl-xL) and Bcl-2.²¹ In highly metastatic prostate cancer cell lines, TIMP-3 was found to promote apoptosis via activation of mitochondrion-mediated caspase-3.²² Additionally, TIMP-3 promotes cancer cell sensitivity to apoptosis via stabilisation of the death receptor.²⁰ When TIMP-3 is transfected into prostate cancer cells, it suppresses cancer proliferation and stimulates apoptosis in nude mice.²³

Metastasis, the main reason for cancer deaths, involves multiple processes, like degradation and remodelling of extracellular matrix (ECM), which is accomplished by different proteinases, including the plasmin system, especially the MMPs, and TIMP-3 can block proteinase activity and suppress invasion and tumour migration.²⁴ The current study revealed that TIMP-3 downregulation caused an increase in the concentration of hepatic MMP-9. A previous study reported that TIMP-3 inhibits the proliferation and migration of smooth muscle cells from the aortic neck during the development of an atherosclerotic abdominal artery aneurysm in rabbits by decreasing the activities of MMP-9 and MMP-2.²⁵ Another study illustrated that TIMP-3 overexpression decreases MMP-2 and MMP-1 in osteosarcoma, and inhibits invasion and cell migration.²⁶ TIMP-3 was also found to protect against the degradation of ECM triggered by ADAM-12, and its expression was correlated with the stage and status of breast cancer (BC).²⁷

Conclusion

TIMP-3 can be used as a promising sensitive marker for early detection and staging of HCC. This may be due to its role in cancer cell growth inhibition and induction of tumour cell apoptosis, and its contribution towards suppressing cancer cell invasion and metastasis.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

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