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Expression of phosphatase and tensin homolog gene in hepatitis C virus induced hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma is a common etiology of cancer-related deaths, and hepatitis C virus is considered a major risk factor. The cutoff level of expression of Phosphatase and Tensin Homolog (PTEN) tumor suppressor gene in predicting hepatocellular carcinoma is still not clearly determined. We aimed to investigate the expression levels of PTEN in diagnosis of hepatitis C-related hepatocellular carcinoma. A group of 50 patients with hepatitis C-induced hepatocellular carcinoma, and a control group of 30 healthy participants were included. History, examination and laboratory investigations were recorded for all the participants. Evaluation of liver disease severity and radiological assessment of hepatocellular carcinoma were done for the patients. PTEN gene expression assay was performed for all participants. Patients had significantly lower levels of PTEN in comparison to the control (0.89 ± 0.23 vs. 2.35 ± 1.09) and PTEN level was lower in advanced liver disease. At a cutoff < 0.67, PTEN had 98% accuracy for diagnosis of hepatocellular carcinoma with area under curve 0.990, while alpha fetoprotein at a cutoff > 155 ng/ml had 69% accuracy with area under curve 0.53. About 45/50 (90%) of patients had down regulation of PTEN gene expression compared to 10/30 (33.3%) of the control. PTEN gene expression level was a significant predictor of hepatocellular carcinoma (p = 0.012). Hepatitis C virus-induced hepatocellular carcinoma was significantly associated with downregulation of PTEN gene expression which increased with advanced liver diseases. At a cutoff of 0.67, PTEN was a significant predictor of hepatocellular carcinoma.

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Introduction

Hepatocellular carcinoma (HCC) is the fourth most common etiology of cancer-related death (Villanueva 2019). Worldwide, Hepatitis B virus (HBV) and hepatitis C virus (HCV) remain the most common risk factors for HCC (McGlynn et al. 2021). Surgical

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treatment is considered the primary management for HCC, and without liver transplantation the 5-year survival rate for patients with HCC is less than 5% (El-Serag et al. 2007; Huang et al. 2022).

A comprehensive understanding of HCC pathogenesis, recurrence and metastasis is mandatory to achieve early diagnosis and development of more effective treatment modalities. The common biomarkers for diagnosis and staging of HCC include alpha-fetoprotein (AFP), des-gamma-carboxy prothrombin (DCP), and lens culinaris agglutinin-reactive AFP (AFP-L3) (Marrero et al. 2009). Each of these biomarkers in HCC diagnosis has its limitations regarding early diagnosis, sensitivity, and specificity.

The tumor suppressor gene phosphatase and tensin homolog (PTEN) gene acts as a tumor suppressor gene, regulating cell division, inducing cell apoptosis and keeping cells from dividing too rapidly or in a controlled manner (Tamguney et al. 2007). PTEN is the second most commonly mutated tumor suppresser gene in cancers after p53 (Peyrou et al. 2015). PTEN regulators include Ras, early growth response protein 1 (EGR-1), reactive oxygen species (ROS), and nuclear factor kappa B (NF-κB)(Kurlawalla-Martinez et al. 2005; Wong et al. 2007).

PTEN gene activities both protein and lipid phosphatase, so it participates in the normal physiological and pathological process of cancers, and correlated with the differentiation, metastasis, and prognosis of tumors (Hu et al. 2007; Vinciguerra et al. 2008).

The major role of PTEN is the downregulation of phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway (Chalhoub et al. 2009). PTEN-mediated suppression of AKT pathway results in an increase in longevity, downregulation of insulin signaling, and tumor suppression (Kang-Park et al. 2003). Recently, deactivation of the PTEN/AKT/mTOR pathway was reported to be involved in the tumor suppressive role of Tripartite motif-containing 29 (TRIM29) gene in HCC (Yin et al. 2024). In addition, PTEN can modulate tumor cell invasiveness by stabilizing E-cadherin/ β-catenin adherens junctional complexes (Kotelevets et al. 2001; Kotelevets et al. 2005), and HCC metastasis though glutathione peroxidase 4 (GPX4) gene (Pan et al. 2024).

PTEN is a target in the pathogenic pathway of HCV core protein and HBV virus X protein which alter PTEN regulation and pro-apoptotic ability to enhance the process of tumor initiation. Therefore, PTEN was considered as a valuable diagnostic and prognostic biomarker in virus- induced HCC (Khalid et al. 2017). Lower PTEN expression correlated with advanced tumor stage and higher expression of AFP (Hu et al. 2003).

In Egypt, HCC represents the fourth common cancer (Akinyemiju et al. 2017). The reason for increased incidence could be attributed to improvement in screening programs and diagnostic tools, increasing the survival rate of cirrhotic patients that increases the chance of developing HCC and increasing the incidence and complications of HCV (Abd-Elsalam et al. 2018) which is the most important risk factor in HCC in Egypt (Rashed et al. 2020; Kobayashi et al. 2022).

Previous studies reported the role of PTEN in HCC in cirrhotic patients secondary to HCV infection, but the exact cutoff level which predicts HCC was not determined. Therefore, the expression of PTEN level in the development of HCC is not yet well-determined. Hence, the current work was conducted to investigate the expression levels of tumor suppressor gene phosphatase and tensin homology (PTEN) in the diagnosis of HCC among patients with chronic HCV infection and comparing its accuracy with AFP.

Materials and Methods

This was a case-control study carried out on patients with HCC on top of liver cirrhosis induced by chronic HCV (cases group), and age- and sex- matched healthy control (control group).

Patients were recruited as out-patients in Hepatoma Clinic in Al-Rajhy Liver University Hospital, and in-patients admitted at Tropical Medicine and Gastroenterology Department, in Al-Rajhy Liver University Hospital during the period from January 2021 to December 2022.

Laboratory analysis of PTEN expression was conducted in the PCR laboratory at Clinical Pathology Department, South Egypt Cancer Institute, Assiut University. Patients were excluded if they have HCC not on top of HCV e.g: HBV, NASH, other malignancies concomitant with HCC, and patients who have previous treatment of HCC by surgery or intervention.

All participants were subjected to a thorough history and clinical examination. The history included age, gender, residence, smoking status, comorbidities such as diabetes mellitus or hypertension, and past and current medications.

Laboratory investigations included a complete blood picture (CBC), liver function tests, coagulation profile, HBsAg, anti-HCV, serum urea, creatinine, alpha-fetoprotein (AFP), and PTEN expression.

Radiological assessment of HCC was performed using abdominal ultrasound and multi-slice triphasic computed tomography (MSCT) to determine the location, number, and size of hepatic focal lesions, as well as to identify portal vein thrombosis, lymph node metastasis or other distant metastases, and ascites.

Assessments of CHILD and MELD scores were conducted using relevant clinical and laboratory data. The MELD score is calculated based on serum bilirubin, serum creatinine, and the international normalized ratio (INR), yielding a number ranging from 6 to 40 that indicates how urgently a patient requires a liver transplant. The CHILD score is an automated calculation based on the presence of ascites, encephalopathy, serum bilirubin, albumin levels, and prothrombin time, and is designed to predict mortality in cirrhotic patients.

Healthy controls were selected randomly matching patients in both age and sex with no history of liver disease or other diseases.

PTEN gene expression assay methods

Three ml venous blood was drawn from an antecubital vein under complete aseptic conditions from each patient and control subject and collected in EDTA tube and then underwent immediate processing for RNA extraction otherwise preserved at (-80^{O}c). Processing and preservation of sample are dealt with under the standard operating procedures of the molecular biology lab, south Egypt Cancer Institute.

Purification of total cellular RNA was done from human whole blood using QIA amp RNA Blood Mini Kit (Germany, cat. No.52304). The QIA amp RNA Blood Mini Kit was stored dry, at room temperature (15–25^{O}C). Reverse Transcription and Quantification was done Using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (K1622, Lithuania). The kit was stored at - 20^{O}C. Detection of PTEN gene by real time PCR was done by using the Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X), ROX Solution provided (K0251) and Applied Biosystems 7500 Fast Real-Time PCR Systems (Thermo Fisher Scientific, USA, serial number 275016697).

For real-time PCR reaction, the following reagents were used: 3 µl cDNA, 1µl of specific of each primer, with a final concentration of 500 n M, 12.5 µl of SYBR Green master mix, 0.05µl Rox solution and 7.45 µl H2O in a total volume of 25 ul. PCR cycling conditions for PTEN and GAPDH amplification were: initial denaturation, 10 min at 95 °C, 45 cycles at 95 °C for 15s for denaturation and 60 °C for 30s for annealing/ extension. The amplification primers used are: For PTEN gene the primer pair forward 5′CCACAGCTAGAACTTATCAAACCCT3′ and reverse 5′TCATTACACCAGTTCGTCCCTTTC3′ and for GAPDH, the primer pair is; forward 5′ACCCAGAAGACTGTGGATGG3′ and reverse 5′TTCAGCTCAGGGATGACCTT3′

Institutional Review Board Statement

This study was approved by the ethical committees of the faculty of science at Al-Azhar University in Assiut (APPROVAL NUMBER / ID: Azhar 10/2024).

Informed Consent Statement

A written informed consent was obtained from all subjects involved in the study.

Results interpretation

For quantitative PCR, the comparative threshold cycle method was utilized for mRNA quantification. The relative expression of a gene is presented by the 2–ΔΔct method where the gene expression level is normalized to an internal control gene (GAPDH) and the cycle threshold (Ct) was determined and was defined as the fractional cycle number at which the fluorescence exceeded the given threshold. Specificity of the product was confirmed by running of melting curve analysis of post PCR reaction

Statistical analysis

Data was collected and analyzed by using SPSS (Statistical Package for Social Science, version 28, IBM, and Armonk, New York). The Shapiro test was used to determine compliance of the data to normal distribution.

Quantitative data with normal distribution are expressed as mean ± standard deviation (SD) and compared with Student t test. Quantitative data with abnormal distribution expressed as median (minimum-maximum) and compared by Mann-Whitney U test. Nominal data are given as number (n) and percentage (%). For comparing categorical data, Chi square ($\chi 2$) test was performed. Fisher Exact test was used instead when the expected frequency is less than 5. Correlation between PTEN expression with other continuous variables was determined by Spearman coefficient correlation. Diagnostic accuracy of PTEN and AFP expressions were assessed by receiver operator characteristics (ROC) curve. Univariable and multivariable logistic regression was performed to determine predictive factors effect on HCC. The level of confidence was kept at 95% and hence, p value was considered significant < 0.05.

Results

This study included 50 cases and 30 healthy controls. Sociodemographic data of the studied groups are shown in table 1 (supplementary). There was no significant difference between both groups regarding age and sex. The mean age of HCC group was 60.56 ± 10.34 years and for control group it was 56.80 ± 9.28 years. The majority of both groups were males, 68.3% in HCC group and 66.7% in control group. Concerning smoking history, there was no significant difference between cases and control groups; however, there was a significant difference between cases and control regarding the comorbidities including hypertension, diabetes mellitus or other chronic diseases. Regarding the laboratory data, table 2 (supplementary) shows that there was a significant difference between both groups in all laboratory parameters except for the albumin and creatinine.

Clinical data concerned including jaundice and encephalopathy which constituted 30% and 4% of patients, respectively. Upon including the radiological findings, it was essentially focused on the focal lesions (location, number and size) (table 3 supplementary). Other radiological findings included are lymph node metastasis which was found in 22 (44%) of cases, portal vein thrombosis in 11 (22%) of cases, and ascites in 15 (30%) of cases. Both MELD & CHILD scores were recorded where child score A was reported in 33 (66%) of cases while CHILD score B was reported in 17 (34%) of cases. Finally, the MELD score was included for cases and has a median of 7.8 and ranged from 3.71 to 12.79

Regarding the PTEN gene expression level, there was a significant difference between patients and controls, where the mean PTEN gene expression value was higher in control than in HCC group (2.35 ± 1.09 vs. 0.89 ± 0.23); $p < 0.001$) i.e. the PTEN gene is significantly down regulated in patients in comparison to the controls as illustrated in figure 1.

Patients with Child class C had significantly lower PTEN in comparison to those with Child class A (0.55 ± 0.10 vs. 0.90 ± 0.80; $p = 0.02$) and those with Child class B (0.55 ± 0.10 vs. 0.80 ± 0.99; $p = 0.01$). However, there was no significant difference between patients with class A and those with class B as regard serum PTEN (0.90 ± 0.80 vs. 0.80 ± 0.99, $p = 0.90$) as demonstrated in table 4 (supplementary).

At cutoff < 0.67 for PTEN gene expression had 98% overall accuracy for diagnosis of HCC with AUC was 0.990 while AFP at cutoff point > 155 ng/ml, it had 69% accuracy for diagnosis of HCC with AUC was 0.53. Hence, the level of PTEN gene expression had better diagnostic accuracy for diagnosis of HCC in comparison to AFP (98% vs. 69%; $p < 0.001$) as seen in table 5 (supplementary).

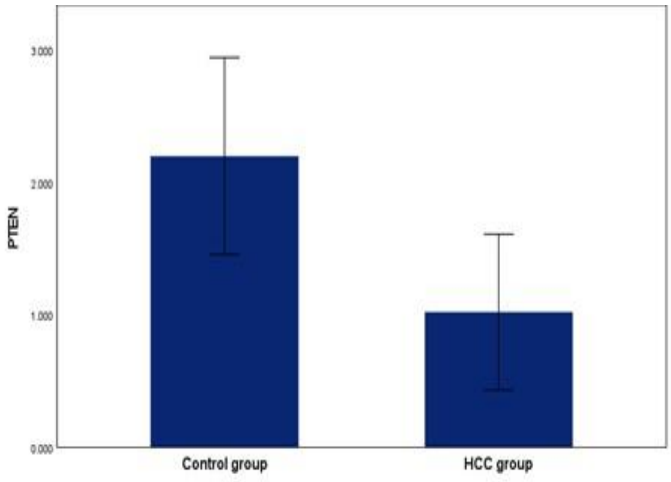

Fig 1. The mean value of PTEN gene expression in the studied groups.

Using the calculated cut off < 0.67 the gene expression value is reclassified as up regulated > 0.67 and down regulated < 0.67. Using these values, it was found that 45 out of 50 (90%) of HCC patients are found to show down regulation of the PTEN gene expression level in comparison to only 10/30 (33.3%) of control group with a highly significant difference $p < 0.001$ as shown in figure 2.

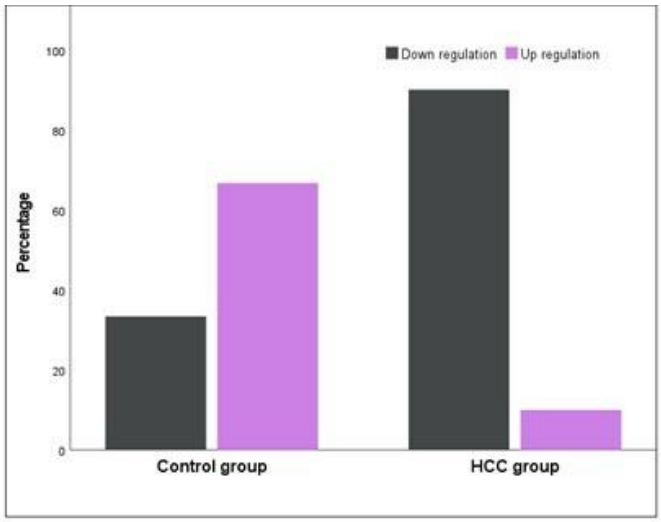

Fig 2. PTEN expression in the studied groups based on the cutoff level.

It was found that PTEN had a significant positive correlation with serum albumin ($r = 0.54$, $p < 0.001$) and negative correlation with AFP ($r = -0.22$, $p = 0.04$), MELD score ($r = -0.49$, $p < 0.001$) and Child score (r =

-0.51, p < 0.001) as illustrated in table 6 (supplementary). Using the univariate model for regression analysis of HCC as an independent factor and examination of other variables, it was found that different variables could have an effect on HCC, however, the PTEN gene expression has a highly significant value (p <0.001) in this context with odds ratio (0.085). Meanwhile, using the multivariate regression analysis model, the PTEN gene expression level still has a significant value in predicting HCC (p = 0.012) with odds ratio (0.006) as shown in table 7 (supplementary).

Discussion

Many tumor suppresser genes and oncogenes are involved in HCC development and have the potential to be used as biomarkers for early detection of HCC and staging of disease. PTEN is one of the most important tumor suppressors in mammals along with p53, Ink4a, and Arf (Buendia 2000; Laurent-Puig et al. 2006; Wang et al. 2019; Chidambaranathan-Reghupaty et al. 2021).

In the current study, we aimed to investigate the expression levels of PTEN in diagnosis of HCC in patients with chronic HCV- related cirrhosis. It was found that both cases and control groups had insignificant differences regarding baseline demographic data, with male predominance in patients with HCC and mean age of 59 year. Consistently with the current study, the incidence of HCC is growing worldwide and increases progressively with advancing age in all populations, with a peak age of 70 years. Males show higher incidence of HCC about 2 or 3 times than females (Sung et al. 2021).

Significantly the incidence and mortality of HCC is much higher in males than females, with greater than threefold difference. Sex hormones act by modulating the risk of developing HCC and affecting its aggressiveness, response to therapy, prognosis, and modulate the action of other factors such as chronic HBV infection or obesity influencing their carcinogenic power (Nevola et al. 2023). Another finding in this study as regard to baseline data is that 46.7% of patients had diabetes mellitus. This higher percentage of patients could be explained by enrollment of all patients with HCV. In line with this study, several reviews and meta-analyses have shown a higher prevalence of comorbidity in patients with HCV than in control (White et al. 2008).

HCV infection has been associated with the development of extra hepatic manifestations, including type 2 diabetes mellitus. The processes by which HCV induces diabetes have not been completely understood, but evidence suggests that insulin resistance is the main mechanism, triggered by several pathogenic causes such as proinflammatory cytokines and chemokines leading to the final impairment of insulin receptor (Persico et al. 2009; Ribaldone et al. 2020).

The main finding in the current study was that patients with HCC had significantly lower levels of PTEN in comparison to the control group. Additionally, the level of PTEN was significantly reduced with late stages of liver disease where patients with Child class A had the highest level of PTEN. Concerning correlation study of the PTEN gene expression with clinical, laboratory and radiological variables, our study revealed a significant fair negative correlation with both MELD & CHILD scores with lower gene expression found in high scores i.e. advanced disease. On The other side, Hu et al. stated that the lower PTEN expression correlated with stage of disease, tumor grade, and higher expression of AFP (Hu et al. 2003; Zhou et al. 2018).

Like other cancers, HCC develops as a result of genetic mutations, leading to an unusual level of cellular replication. It causes the cell to avoid the process of apoptosis. In these types of cells, the damaged DNA continues to multiply and increase in number with the mutated DNA copies. As these cancerous cells reproduce, they start to form tumors which may be malignant or benign (Rivlin et al. 2011; Kan et al. 2013).The first evidence supporting a critical role for PTEN in liver cancer came from genetic studies in mice, where heterozygous deletion of PTEN was shown to induce atypical adenomatous liver hyperplasia (Podsypanina et al. 1999). Additional studies demonstrated that PTEN deficiency in the liver induces hepatomegaly and HCC (Horie et al. 2004; Stiles et al. 2004).

The possible explanation reported by Zhang et al where they found that, HCV is the major cause of HCC, and HCV core protein results in reactivation of the downstream PI3K/AKT signaling pathway; hence, the normal negative regulatory mechanism of PTEN gene for cell division and metastasis will be lost, and malignant transformation of tumors will be accelerated (Zhang et al. 2014). Therefore, targeting PI3K/AKT/mTOR which is regulated by PTEN gene can be considered as an emerging therapeutic approach for HCC (Pessino et al. 2024), also novel biomarkers might play an important role in the screening and prognosis of HCC as a new clinical method to detect PTEN mutation such as CRISPR-Cas12a (Hu et al. 2024). Recently, PTEN deficiency was also reported to potentiate HBV-associated liver cancer development (Huang et al. 2024).

Previous studies showed that PTEN is involved in HCV-induced hepatic dysfunction, and that low PTEN protein activity is associated with the incidence of several hepatic pathologies such as liver cancer and cirrhosis (Peyrou et al. 2010; Li et al. 2015). The diminished production of PTEN protein in human HCC is associated with more aggressive tumor behavior, larger tumor size and mortality (Sze et al. 2011). In addition to its own down regulation, loss of PTEN expression is also related to increased expression of CD133, CD90, and epithelial cell adhesion molecule, all of which are markers of liver cancer. Down regulation of PTEN had a strong prognostic role as a marker for HCC recurrence and shorter survival times (Su et al. 2016).

Another study conducted in China observed that overall, 50% of the HCC samples had no expression of PTEN protein (Wan et al. 2003). Comparison between PTEN expressions in resected HCC liver tissue and surrounding parenchyma also reveals interesting findings. Rahman et al. found that although surrounding normal liver parenchyma had strong expression of PTEN in 90% of the cases, there was low expression of the protein in 65% of the tumorous liver tissue (Rahman et al. 2002). Meanwhile, loss of PTEN expression was observed in 36% of liver tumors compared to normal tissue and level of PTEN expression was inversely correlated with the tumor stage (Chen et al. 2009).

To the best of our knowledge, this is the first study that determines a cutoff point for PTEN expression level to predict HCC in cirrhotic patients secondary to HCV infection. At cutoff < 0.65, PTEN had 98% overall accuracy for diagnosis of HCC with AUC was 0.990. For diagnosis of HCC, serum PTEN had better diagnostic accuracy for diagnosis of HCC in comparison to AFP (98% vs. 69%; $p < 0.001$). In multivariate analysis in the current study, it was found that PTEN was a significant risk factor for HCC.

The present study had some limitations. Firstly, the relatively small sample size and the conducted study is single center, secondly, all enrolled patients had viral hepatitis with exclusion of other etiologies of HCC and lastly, we did not perform survival analysis in this study to assess the correlation of PTEN with survival of those patients and assess its relationship with the recurrence and progression of HCC. However, our study considered the first study that determined a cutoff point for PTEN to predict occurrence of HCC in patients with liver cirrhosis secondary to HCV infection.

Conclusion

HCV-related HCC was significantly associated with downregulation of PTEN tumor suppressor gene. At a cutoff point 0.67, PTEN was a predictor of HCC. Moreover, PTEN showed a higher accuracy than the commonly used tumor marker AFP in predicting HCC. However, further studies on a larger number of patients in multiple centers are required to confirm and provide generalizability of the results of this study.

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Conflict of interest

Authors declare they have no known conflict of interest.

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