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# Interference between miR-21/PTEN/E-Cadherin and Epithelial-Mesenchymal Transition in Various Stages of Chronic HCV Infection

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**Running Title**: Influence of mir-21/PTEN/E-Cadherin on EMT in HCV patients

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## Abstract

Hepatocellular carcinoma (HCC) is a major complication associated with hepatitis C viral infection (HCV). The epithelial-mesenchymal transition (EMT) is critical in HCC invasion and metastasis. Several microRNAs (miRNAs) have been linked to HCV-related HCC. This study aimed to evaluate the relation between miR-21, phosphatase, tensin homolog deleted on chromosome ten (PTEN), and E-Cadherin with a flashlight on their role in the EMT process in HCV infection at different stages. One hundred HCV-infected patients were studied, 75 had HCV-induced cirrhosis (classified into Child A, B, and C), and 25 had HCC. In parallel, 45 healthy volunteers were considered normal controls. Circulating miR-21was detected by quantitative Reverse Transcription Polymerase Chain Reaction (qRT–PCR).PTEN and E-cadherin serum levels were measured using Enzyme-Linked Immunosorbent Assay (ELISA). A significant elevation in miR-21 was observed in HCC patients compared with control ones($P<0.01$). HCC patients had the lowest E-cadherins and PTEN ($P<0.01$) compared with cirrhotic and normal subjects. In HCC patients, PTEN was positively correlated with E-cadherin ($r= 0.501$; $p<0.01$). On the other hand, a negative correlation between miR-21 and both E-cadherins ($r= -0.455$; $p<0.01$) and

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PTEN (r= -0.255; p<0.05) was observed. Accordingly, up-regulation of miR-21 in the tumor is an important step in HCV-positive cirrhotic hepatocarcinogenesis and might result in concomitant down-regulation of PTEN and E-cadherin in favor of tumor promotion. Our data might be the first study that correlates miR-21, PTEN, and E-cadherin in different stages of HCV infection (from cirrhosis to HCC).

**Keywords:** miR-21, PTEN, E -cadherins, HCV, HCC.

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## 1. Introduction

Hepatitis C virus represents the most common blood-borne hepatotropic pathogen and a leading cause of morbidity and mortality worldwide [1, 2]. It has six major genotypes (types 1–6); genotype-4 represents 12%–15% of total HCV infection [3]. Globally, Egypt has the highest prevalence of HCV infection. About 90% of Egyptian patients suffering from HCV belong to genotype-4 [4, 5]. Based on the last World Health Organization (WHO) global hepatitis report [6], ~1% of the world's population was infected by HCV. Acute HCV infection results in spontaneous viral clearance ~12 months post-infection. Otherwise, HCV-infected patients develop a chronic hepatitis C (CHC) infection that can lead to liver cirrhosis and, eventually, hepatocellular carcinoma (HCC) [7, 8].

HCC is one of the most frequently diagnosed cancers worldwide, accounting for 70–85% of all liver cancer cases [9, 10]. In Egypt, it is the second leading cause of cancer-related death in males and the fifth in females, and it's continued to be [11, 12]. HCC is associated with poor prognoses because of its aggressive growth, metastasis, and resistance to most current therapeutic approaches [13]. Until now, there is no effective treatment, especially for advanced-stage cases.

Epithelial-mesenchymal transition (EMT) plays a vital role in invasion and metastasis in diverse types of cancer, including HCC [14, 15]. During EMT, cancer cells lose their epithelial characteristics and acquire entirely mesenchymal phenotypes. Downregulation of epithelial markers is one of the trademarks of EMT. According to growing evidence, EMT is becoming increasingly crucial in HCC invasion and metastasis [16, 17]. Several factors have been identified as master regulators of EMT, including cadherins, a transmembrane glycoprotein that mediates calcium-dependent cell-cell adhesion. Changes in cadherin expression are linked to EMT during cancer metastasis [18, 19]. Neural cadherin (Ncad) and epithelial cadherin (Ecad) are the Classical type I cadherins that function to link to the actin cytoskeleton and intracellular signaling pathways [20, 21]. E-cadherin and N-cadherin act as crucial regulators in the process of tumor development. E-cadherin is essential in maintaining epithelial tissue integrity and providing strength to preserve the polarization of the epithelial cell layers. N-cadherin is highly expressed in mesenchymal cells and neural tissue [22]. N-cadherin promotes increased cell motility and migration [23]. During malignancy, cell-cell adhesion mediated by E-cadherin is lost [24], and tumor cells transformed from normal epithelium to motile mesenchymal and acquire more N-cadherin in a process known as cadherin switch [25].

A major epigenetic factor controlling EMT is MicroRNAs (miRNAs), a group of non-coding single-stranded short RNAs (18–22 nucleotides) that attach to the 3'-UTR of target mRNA to decrease gene expression. Many studies have recently reported that miRNAs play a key role in EMT regulation [26]. miR-21 has been identified as a typical example of oncomir [27, 28]. Oncogenic miRNAs are known to upregulate mesenchymal proteins and down-regulate epithelial molecules. Thus, miR-21 promotes EMT as it

contributes to the loss of epithelial markers, such as E-cadherin, and the acquisition of mesenchymal markers, N-cadherin [29, 30]. A study by Emerling et al. [31] indicated that miR-21 gene editing and silencing might inhibit the phenomenon of EMT and cancer progression.miR-21 regulates the expression of tumor suppressor genes such as phosphatase and tensin homolog (PTEN). Via suppressing PTEN, miR-21 can increase hypoxia-inducing factor (HIF-1), which directly affects the expression of many EMT regulators and induces the repression of E-cadherin in cancer cells [32].miR-21 is negatively correlated with PTEN expression in many tumors [33].

Alterations of the different miRNAs in HCV patients have been related to either malignancy (HCC), liver cirrhosis, or both [34]. Moreover, miR-21 was rapidly upregulated following HCV infection [3, 33]. It is over-expressed in HCV-positive liver biopsy samples [35, 36, 37]. miR-21 was found to be more strongly expressed in HCC specimens than in non-tumorous tissues [38], and it could be considered a promising biomarker and treatment target in HCC [39-41]. In liver cancer, the elevation of miR-21 in liver cancer coincided with a reduction in PTEN expression [42, 43].

To the best of our knowledge, no previous study was conducted on the relation between these triangle parameters (miR-21, PTEN, and cadherins) in HCV-related HCC patients. Thus, we carried out this work to investigate their circulating expression level in HCV-infected patients with different clinical manifestations (from cirrhosis to HCC).

## 2. Patients and Methods

### 2.1. Patient and clinical samples

The study population was collected from Clinical Pathology Department, National Liver Institute, Menoufia University, Egypt. The local Ethics Committee and Institutional Review Board approved the study protocol. All investigations were carried out by Menoufia University's Health and Human Ethical Clearance Committee criteria for clinical studies.

A total of 145 participants were included in our study; 100 HCV-infected patients, 75 of whom had cirrhosis, and 25 had HCC. Forty-five 45 age-matched healthy volunteers who went to the hospital blood bank after donating blood served as normal controls with standard liver function tests and no history of previous liver disease and negative HBV and HCV serology.

The included patients must be HCV-PCR positive by TaqMan HCV assay (Roche instrument center AG, Switzerland) and positive HCV antibody by third-generation enzyme-linked immunosorbent assay (ELISA). All patients were subjected to complete clinical examinations and routine laboratory investigations. The investigations include Complete blood picture (CBC), prothrombin time, liver function tests (alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and bilirubin, alkaline phosphatase (ALP), alpha-fetoprotein (AFP)).

Clinically, cirrhotic patients have varices, splenomegaly, or a small liver size with a rugged liver surface. The Child-Pugh scoring method was used to categorize cirrhotic patients [44] into Child A (mild) with a total score of 6, Child B (moderate) with a total score of 7-9, and Child C (severe) with a total score >9. In addition to elevated serum AFP levels, at least two radiological tests were used to diagnose HCC patients: abdominal ultrasound, magnetic resonance imaging (MRI), hepatic angiography, and contrast-enhanced dynamic computed tomography (CT). Co-infections with the hepatitis B virus (HBV) or HIV, organ transplantation, immunosuppression, autoimmune illness, diabetes, Schistosomiasis, and other malignancies were all ruled out. Patients under antiviral medication or chemotherapy were also excluded.

For all individuals in this study, 5 ml of venous blood were withdrawn, with 2 ml going into an EDTA-containing tube for miRNA detection and 3 ml going

into a gel tube for biochemical analysis. For ten min., all tubes were spun at 4000 rpm. The serum and plasma fractions were isolated and kept at -80°C until they were needed.

### 2.2. miR-21 Expression by Quantitative Real-Time Reverse-Transcription Assay (qRT-PCR)

Total RNA from 200 $\mu$l plasma samples was isolated using miRNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The purity and concentration of extracted RNA were assessed by NanoDrop Spectrophotometer (NanoDrop ND-1000, United States). For a quantitative analysis of miRNA-21, a two-step real-time PCR analysis was performed using primers [hsa-miR-21-5p (MS00009079) and RNU66 (internal control; MS00033740)] obtained from Qiagen. cDNA was synthesized from total RNA using miScript II RT Kit. Amplification and quantification of miR- 21 were done using miScript SYBR Green PCR kit (Qiagen) in AriaMax Real-Time PCR (Agilent) according to manufacturer instructions. miR-21 expression levels were determined using the equation $2^{-}\Delta\Delta$ct method ($\Delta$CT = CTmiR-2 - CTU6) as previously described [25].

### 2.3. Measurement of plasma E-cadherin and PTEN

E-cadherin and PTEN plasma levels were measured in all patients and normal controls by sandwich enzyme-linked immunosorbent assay (ELISA). Total concentrations of E-cadherin and PTEN were measured using ELISA (INTRON, Bioneavan Co., Ltd China). Using a UV-max ELISA plate reader (SunriseTM), absorbencies were measured at 450 nm. The raw absorbance readings' digital data was processed into a standard curve by the ELISA reader-controlling software (Softmax). The E-cadherin and PTEN concentrations of the samples were determined. The results were shown as a pictogram per milliliter (pg/ml).

### 2.4. Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Science version 19 (SPSS, Inc., Chicago, IL). Clinical data were statistically described as mean ± standard deviation (M ± SD), frequencies when appropriate. One-way variance analysis (ANOVA) was used to compare various groups, followed by the Tukey as a post-doc test. Using the Chi-square measure, the frequency was compared. Receiver Operating Characteristic [ROC] curves were constructed to define the optimal sensitivity and specificity of the miR-21 expressions. Correlation between variables was determined using Pearson's correlation test. The level of significance was $P<0.05$.

## 3. Result

### 3.1. Clinical and laboratory characteristics of patients and controls

The demographic and clinical data of all studied patients and control are summarized in **Supplementary Tables (1)**. We enrolled 100 HCV patients in this study, 75 had cirrhosis, and 25 had HCC. Cirrhotic patients were subdivided into 3 Child groups; A, B, and C (25 each). Forty-five normal healthy controls were run in parallel. All patients showed significantly increased serum AFP levels compared to controls, with maximum production predicted in HCC patients.

Clinicopathological examination of HCC patients showed the presence of focal lesions either in the right (72%) or left (28%) lobe of the liver with a 3.08±1.25 mean focal lesions number. Ten patients (40%) have single focal lesions, while 15 (60%) have multiple. Concerning the tumor size, CT data revealed that 14 HCC patients (56%) have tumor size >3 cm while the other 11(44%) have 3-6 cm tumors.

### 3.2. miR-21 expression levels

Comparable levels of miR-21 were found in all stages of cirrhosis. HCC patients showed a significant increase in miR-21 ($P<0.01$) expression levels

compared to normal subjects **(Figure 1)**. On the contrary, HCV-infected patients at different stages of cirrhosis have a significant reduction in miR-21 expression levels concerning HCC counterparts (P<0.01; P<0.01; P<0.001 for Child A, B, and C; respectively).

Based on ROC curve analysis (**Figure 2**), the area under the curve (AUC) for miR-21 was 0.682 (95% CI: 0.561-0.813) and for AFP was 0.930 (95% CI: 0.846-1). A cutoff value of 0.76pg/ml was chosen with a sensitivity of 80% and specificity of 57%t for miR-21. A cutoff value of 8.5 pg/ml was selected with a sensitivity of 88% and specificity of 100% for AFP.

### 3.3. E-cadherin and PTEN levels

As shown in Figure (3), consistent levels of PTEN in all cirrhotic patients were found. A significant reduction in PTEN level was found in HCC patients (P<0.01) compared with normal controls. The maximum elevation of PTEN was observed in Child B patients. The same results were observed in E-cadherin (Figure 4), with a significant maximum of production in Child B cirrhotic patients (P<0.01) and significant diminution in HCC patients (P<0.01).

### 3.4. Correlation between miR-21, PTEN, and E cadherins

As illustrated in Figure (5), miR-21was negatively correlated with both E-cadherin (r= -0.455; p<0.01) and PTEN (r=-0.255; p<0.05) in HCC patients. On the other hand, PTEN was positively correlated with E-cadherin (r= 0.501; p<0.01).

**Supplementary Table (1).** Demographic and clinical parameters in HCV infected patients and normal controls

| Parameter | Control (N=45) | Cirrhosis (N=75) | Child A (N=25) | Child B (N=25) | Child C (N=25) | HCC (N=25) | P |
|---|---|---|---|---|---|---|---|
| **Age** | 48.29±6.85 | 56.02±12.19 | 49±13.67 | 54.52±9.09 | 64.56±7.71 | 56.24±5.75 | NS |
| **ALT** | 17.09±5.79 | 56.02±12.19 | 24.68±15.72 | 53.88±68.38 | 49.44±45.48 | 54.28±28.35 | P<0.001 |
| **AST** | 20.93±6.11 | 42.66±49.34 | 25.44±17.08 | 47.28±36.68 | 87.36±89.23 | 67.76±40.08 | P<0.001 |
| **ALB** | 4.3±0.47 | 5.36±61.48 | 4.3±0.52 | 3.1±0.52 | 2.28±0.38 | 3.4±0.71 | P<0.001 |
| **TBIL** | 0.62±0.29 | 6.58±1.32 | 0.6±0.25 | 4.04±6.27 | 7.14±7.27 | 1.32±0.7 | P<0.001 |
| **DBIL** | 0.09±0.11 | 3.92±6.09 | 0.22±0.12 | 3.01±5.44 | 5.64±6.56 | 0.51±0.53 | P<0.001 |
| **INR** | 1±0.1 | 2.96±5.34 | 1.13±0.08 | 1.35±0.18 | 1.56±0.35 | 1.17±0.11 | P<0.001 |
| **AFP** | 3.32±1.05 | 1.34±0.29 | 4.45±1.9 | 20.25±26.42 | 166.44±679.45 | 1229.13±3501 | P<0.01 |

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ALB: Albumin; TBIL and DBIL: Total and direct bilirubin, INR: international normalized ratio; AFP: alpha-fetoprotein.

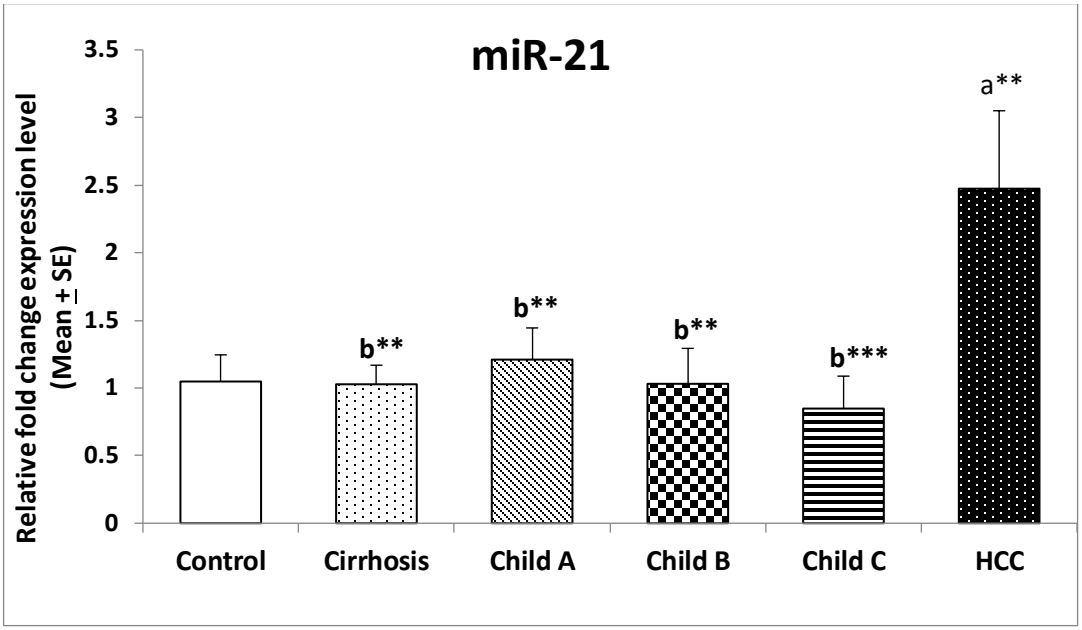


**Figure (1).** The relative expression level of the circulating microRNA-21 in HCV-infected patients' different clinical manifestations and normal controls. (a) Statistically significant from the control group; (b) Statistically significant from the HCC group. (**) P<0.01; (***): P<0.001.

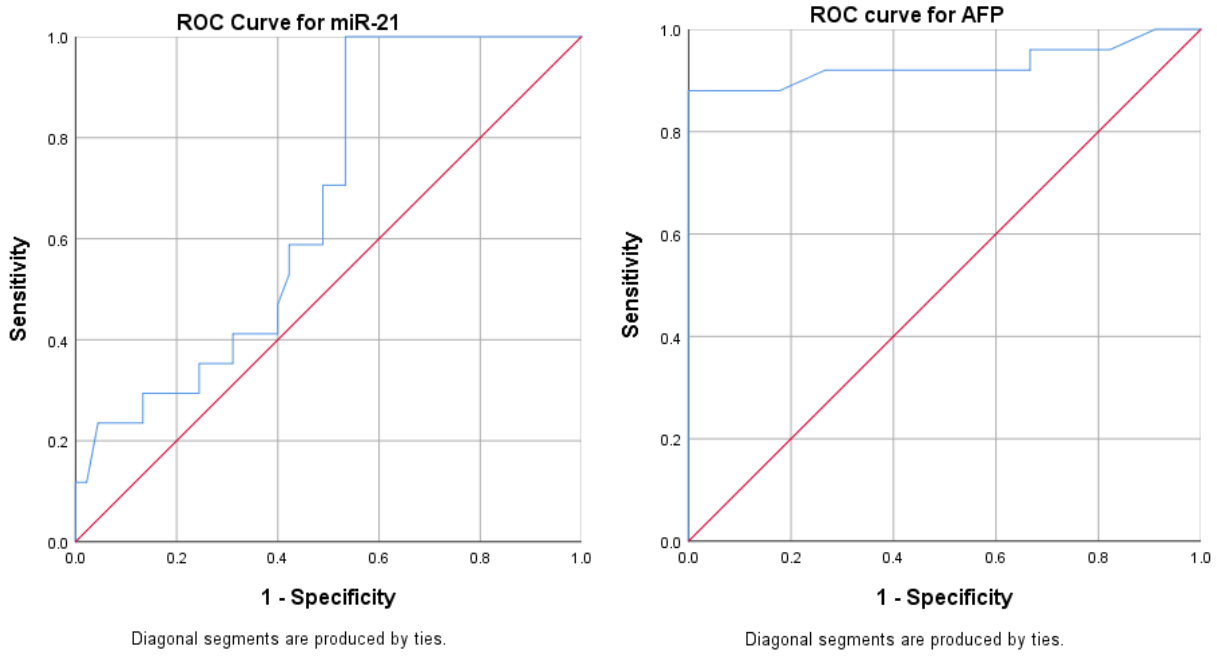


**Figure (2):** ROC Curve of miR-21, AFP, and combined miR-21 and AFP.

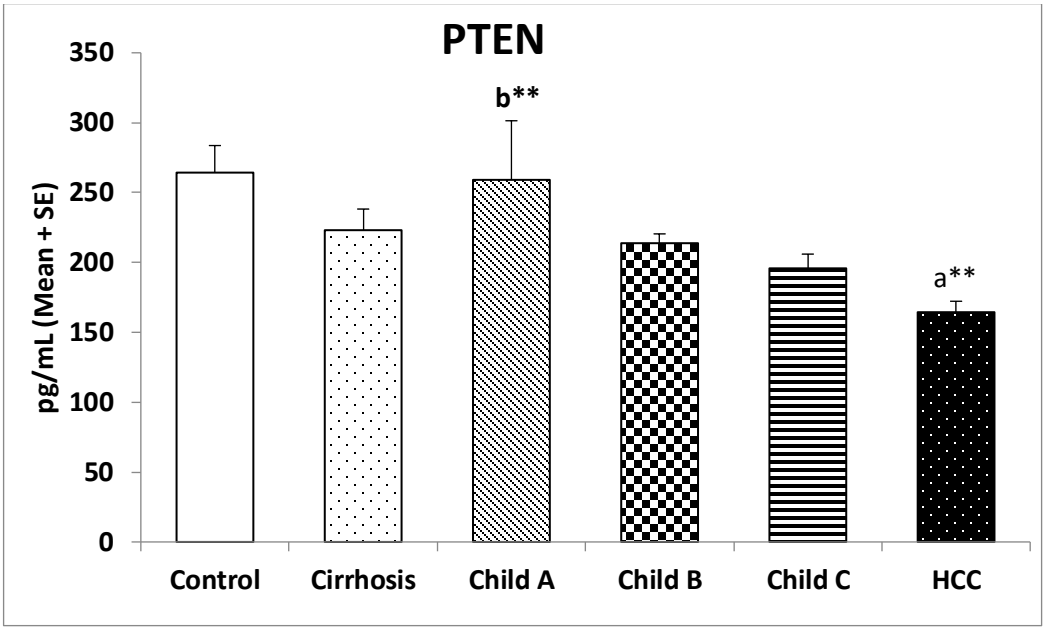


**Figure (3)**: Circulating PTEN in HCV-infected patients at different clinical manifestations and normal controls. (a) Statistically significant from the control group; (b) Statistically significant from the HCC group. (**) P<0.01.

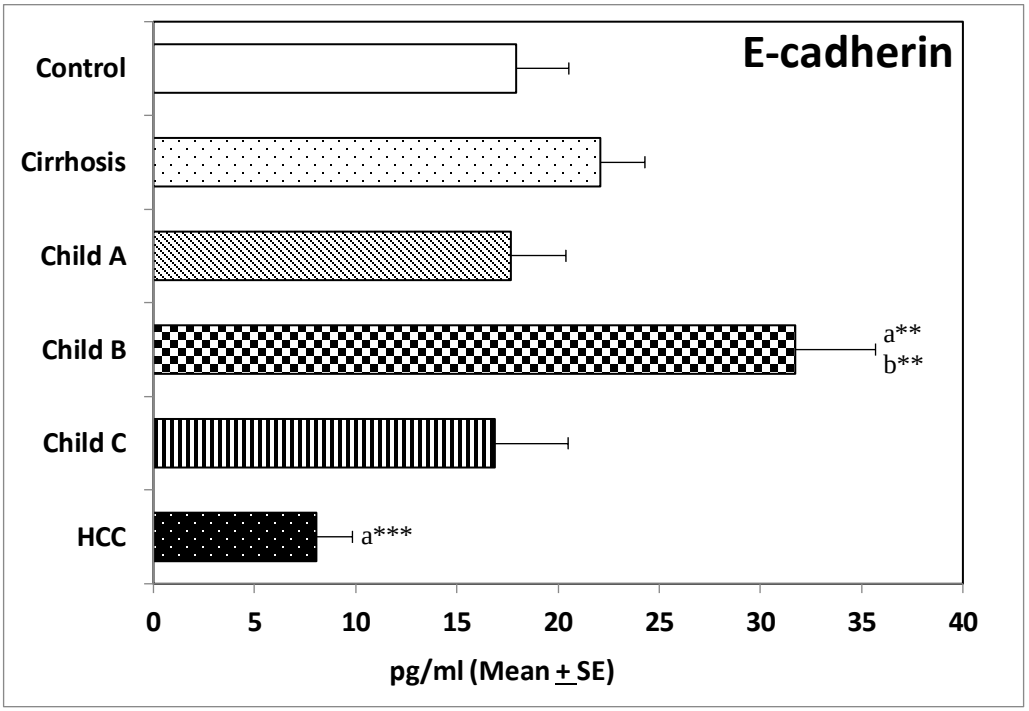


**Figure (4):** Circulating E-cadherin in HCV-infected patients at different clinical manifestations and normal controls. (a) Statistically significant from the control group; (b) Statistically significant from the HCC group. (**) P<0.01; (***): P<0.001.

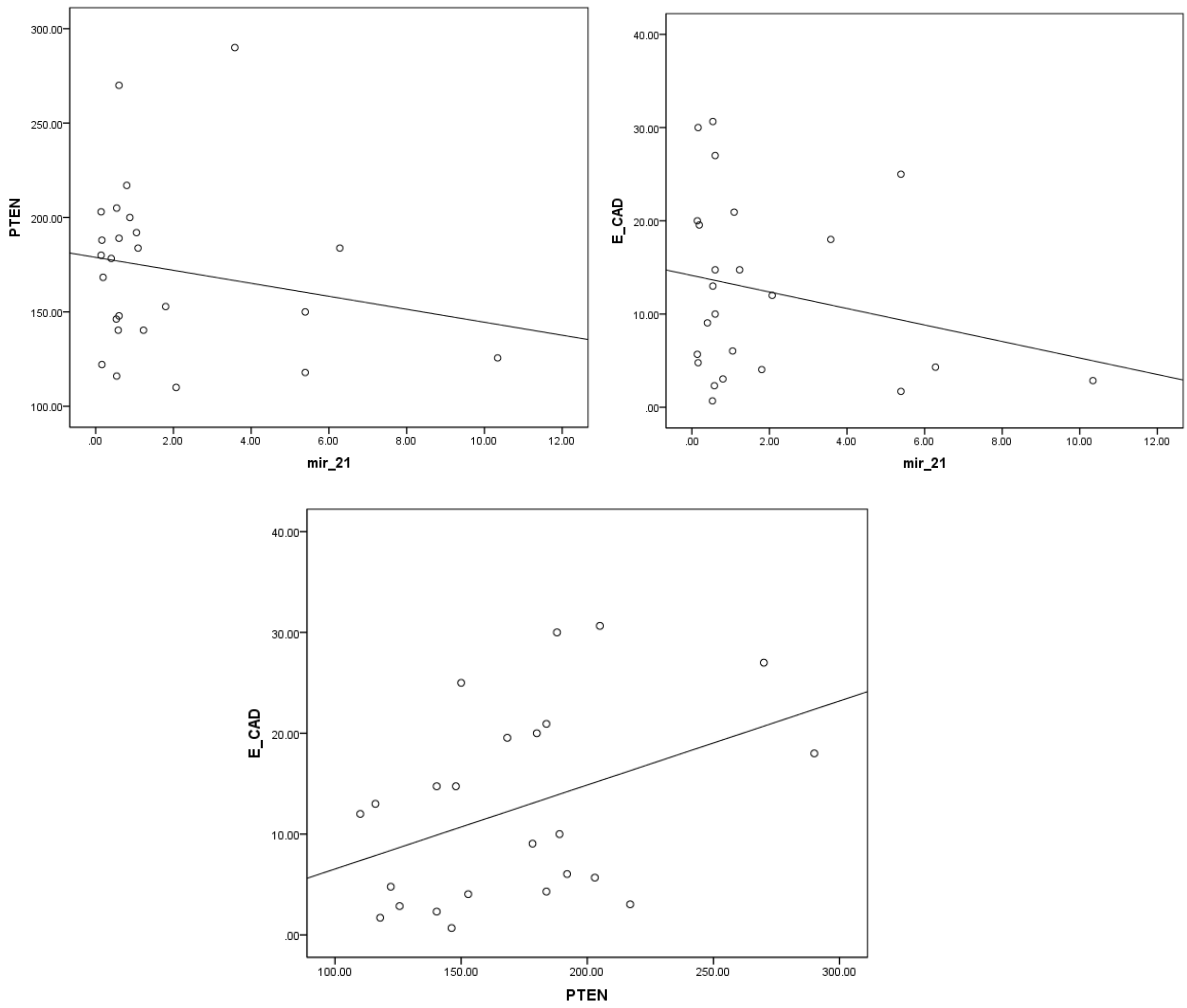


**Figure (5):** Correlation between miR-21, PTEN and E-cadherins in HCV-related HCC patients.miR-21 was negatively correlated with both E-cadherin (r=-0.455; p<0.01) and PTEN (r= -0.255; p<0.05) while PTEN was positively correlated with E-cadherin (r= 0.501; p<0.01).

## 4. Discussion

The activation of EMT and abnormal production of miRNAs in malignancies, including HCC, have been linked to tumorigenesis, tumor development, metastasis, and relapse [45-47]. Besides miRNAs, inducers of EMT include some proteins, cell adhesion molecules, and growth factors. The present study investigated the relationship between miR-21, PTEN, and E-cadherin in HCV-infected patients with different clinical manifestations with quiet insight into their role in the EMT process in HCC.

As shown in our results, the expression level of miR-21 was significantly increased in HCC patients compared to healthy controls. Our data agree with the previously reported study of Lendvani et al. [48], who proved that the expression level of miR-21 is significantly elevated in HCC patients. miR-21 is also overexpressed in hepatic tissues from HCV-infected patients [49-52] and hepatic cancer cell lines [53]. Our data

agreed with Wang et al. [54] data on the same track. They documented an increase in miR-21 expression level in HCV patients, which could be evolved from tissue injury caused by hepatitis infection. Clément et al. [51] prove that HCV-activation of miR-21 is a crucial molecular step that promotes the HCV life cycle. Agreeing with our results, Nasser et al. [48] showed that HCC patients had significantly higher serum miR-21 1evels than non-HCC patients (non-cirrhotic and cirrhotic groups). Accordingly, along the spectrum of HCV-related chronic liver disease, miR-21 could be a helpful biomarker and prognostic factor for HCC. Moreover, significantly higher serum miR-21 has been reported in patients with liver cancer and patients with many kinds of malignancies [55, 56].

miR-21 is an essential component involved in the cellular signaling pathways that regulate EMT processes [45, 57]. It has been shown to play a critical role in the processes of EMT of cancer cells to promote the progression and metastasis of human malignancies, including HCC [46, 47, 58-60]. A tumor suppressor, PTEN, is a typical target gene of miR-21 [61]. Some studies have demonstrated that PTEN is a significant inhibitor of EMT, confirmed in vitro and experimental models in HCC [62, 63]. Our research revealed that up-regulation of miR-21 expression in HCC patients correlated with decreased PTEN production levels. Injection of synthetic miR-21 inhibitors (antagomiRs) was suggested to reduce tumor occurrence and growth in hepatocyte-specific PTEN knockout mice [64]. Our result agrees with Clémentet al. [51], who observed that the secretion level of PTEN was decreased in HCC patients. Cao et al.[65]agreed with our results as they diminished the secretion level of PTEN in HCC patients. It may be explained that the elevation in miR-21 can decrease the expression of PTEN, promote proliferation and migration, and inhibit the apoptosis of HCC cells. PTEN was identified as a negative switch of the protein kinase B (Akt) pathway. Its down-regulation can activate the AKT pathway, thus contributing to the aggressive progression of HCC [66]. Subsequently, deregulation of the miR-21/PTEN signaling axis likely represents a pathological mechanism shared by all chronic liver disorders and, therefore, is highly relevant for therapeutic targeting [51].

The significant changes in gene expression profile occurring in EMT are associated with decreased expression of epithelial genes such as E-cadherin, which is partially or wholly lost during carcinogenesis and advancement of malignancy [67]. Previous studies found that E-cadherin is down-regulated in many types of epithelial malignancies such as HCC [68, 69]. In the current study, a significant decrease in expression of E-cadherin in HCC patients compared to normal subjects was observed. Our result was in agreement with Kasprzak et al. [70], who found a reduction in E-cadherin in HCC patients. The loss of expression of E-cadherin in epithelial cells is a major molecular event in EMT. Several transcription factors mediate E-cadherin's repression [71, 72] as stabilization of E-cadherin at the adherent junction is critical for maintaining the epithelial phenotype [73].

This study found that the up-regulation in miR-21 is significantly correlated with low E-cadherin and PTEN in HCC patients. These factors (miR-21, E-cadherin, and PTEN) are closely associated with EMT. The changes in gene expression profile occurring in EMT are associated with decreased expression of epithelial genes such as E-cadherin. miR21, via suppressing PTEN, increases the phosphatidylinositol 3,4,5 triphosphate (PIP3) and HIF-1α [31]. It is known that HIF-1α directs the expression of many EMT regulators and induces the loss of E-cadherin by transcriptional activation of genes encoding repressors of E-cadherin expression [32].

In conclusion, we investigated the expression patterns of EMT-related genes (miR-21, PTEN, and E-cadherin) in HCV-associated HCC patients and their relationships with one another. In this study,

upregulated miR-21 was linked to chronic HCV infection and eventual complications of carcinogenesis. This could indicate that persistent HCV infection changes the expression of numerous proteins, including PTEN and E-cadherin, driving normal hepatocytes to malignancy via miR-21. Our findings could lead to new targets for preventing and treating HCC metastasis. Further studies are needed using human malignant tissues to improve our results.

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6. **Conflict of interest:** Authors declare no conflict of interest

7. **Reference**

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