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Fibronectin's Footprint in Navigating Hepatocellular Carcinoma Progression

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Abstract

Examining the intricate changes in the extracellular matrix (ECM) dynamics has become a focal point in our ongoing exploration of carcinogenesis. Our study aims at analysis of fibronectin (FN), a pivotal ECM protein, during hepatocellular carcinoma (HCC). A comprehensive analysis combining clinical and statistical methodologies identified elevated FN levels in HCC patients, exhibiting a 2.1 and 1.7-fold increase compared to fibrotic and cirrhotic patients, respectively. Moreover, the area under receiver operating characteristic curve (AUC=0.89) of FN displayed its ability to distinguish between HCC and cirrhosis patients with specificity of 80.4% and sensitivity of 83.7%. Furthermore, ROC curves of FN displayed an AUC of 0.93 with specificity of 86% and sensitivity of 83.7%, this was for separating all non-cancerous patients from HCC. Additionally, HCC patients demonstrated distinctive histological features such as vascular invasion, nodules count, and tumor size, underscoring FN's substantial role in HCC progression. Our findings highlight the significant involvement of FN in the development of hepatocellular carcinoma.

Keywords: Hepatitis C virus, cirrhosis, HCC, ECM, Fibronectin.

Introduction

Hepatitis C virus (HCV) is a challenging health problem, and since this virus affects mainly the liver; its persistence in liver cells can develop into a chronic liver disease, which in turn promotes serious health defects such as: fibrosis, cirrhosis, and eventually liver cancer can be developed (**Pol *et al.*, 2021**). The most common type of primary liver cancer is hepatocellular carcinoma (HCC); accounting for nearly 75-85 % of liver cancer cases (**Chartampilas *et al.*, 2022**). It is the fourth reason resulting in cancer associated death around the world and is ranked 6th diagnostically (**Koulouris *et al.*, 2021**). In Egypt, HCC is considered the fourth widespread cancer (**Rashed *et al.*, 2020**). The

insufficiency of HCC screening among high risk (cirrhosis) patients is indeed a milestone contributor to the predominance of this cancer. Whereas, number of United States and European studies implicated that lower than 40% of cirrhotic patients receive convenient screening (Goldberg *et al.*, 2017). As such, it is of paramount significance to explore novel appropriate biomarkers to detect HCC in patients with bad prognosis (Parikh *et al.*, 2020).

One vital and essential modification in the liver environment leading to cancer progression is the modulation of extracellular matrix (ECM); a prospective with evidential substantiation (Theocharis *et al.*, 2019). Whereas HCC microenvironment is made up of cirrhotic tissues and significant amount of ECM deposit. This in turn leads to several fundamental alternations of the microenvironment; including enhanced matrix stiffness, and solid stress (Poole *et al.*, 2019). Over secretion of remodeled ECM proteins in cancerous microenvironment can be used as a motivation to examine the correlation of this incidence with diagnostic outcomes of the disease. An important component of ECM is fibronectin (FN) protein, which is involved in some pathological incidents such as malignancies (Dalton and Lemmon, 2021). Additionally, several studies have reported that secretion of FN variants has been noticed in different sorts of cancers involving liver cancer, and that FN has diverse roles in the process of propagation, migration, and metastasis of the cancer cells (**Wang *et al.*, 2021**). Hence, we aimed to assess FN as a potential biomarker in HCC histological progression among chronic hepatitis C (CHC) patients.

Materials and methods

Patients

The present study concluded 445 CHC patients; 127 females with mean ± standard deviation (SD) age of 49± 11.4, and 318 males with mean ± standard deviation (SD) age of 50.1± 9; they were enrolled from Tropical Medicine Department, Mansoura University Hospital, Mansoura, Egypt. All patients were negative for HBsAg and for other pathologies causing chronic injury of the liver. Patients with HCC were positive for HCV RNA by polymerase chain reaction (PCR) (COBAS Ampliprep/ COBAS TaqMan, Roche Diagnostics, Pleasanton, U.S.A.). Liver biopsies of all patients were collected randomly by a surgeon using an18-guage or larger needle, size was at least 15 mm with minimum 5 portal tracts; to be proper for scoring. These biopsies were taken and handled to be used for METAVIR. All individuals included in this study provided written assent to collaborate and the study system was ethically scheduled to the 1975 Helsinki guideline (Shephard, 1976).

Classification of patients

METAVIR score system was used to classify patients according to fibrosis status. This score system consists of 5 scores; score 0: no fibrosis, score 1: fibrosis only, score 2: fibrosis with occasional septa, score 3: fibrosis with several septa and occasional nodules, score 4: cirrhosis (bridging with nodular regeneration) (Group and Bedossa, 1994, Bedossa and Poynard, 1996). Accordingly, patients were divided into 3 groups: fibrotic liver patient group (F1-F3) (165 patient) with mean ± standard deviation (SD) age of 42.9±8.6 years; 114 male, and 51 female, cirrhotic liver patient group (F4) (163 patient) with mean ± standard deviation (SD) age of 51.1±9.9 years; 111 males, 52 females, and HCC patient group (117 patient) with mean ± standard deviation (SD) age of 57.2±8.9 years; 93 male, 24 female.

Laboratory methods

Blood samples were collected in EDTA-K3 tubes and run through KX-21 Sysmex automated hematology analyzer (Sysmex Corporation, Kobe, Japan) to count blood cells involving platelets. Other blood samples were collected and centrifuged for sera preparation to be analyzed for some liver function tests, using spectrum commercial kits according to colorimetric method (Egyptian Company for Biotechnology - Spectrum Diagnostics (S.A.E)), such as: albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin which were performed by an automated biochemistry analyzer (Hitachi 917; Roche Diagnostics, Mannheim, Germany). Level of α-fetoprotein (AFP) was determined by the help of chemiluminescence, using IMMULITE (1000) AFP kit (Diagnostic Products

Corporation, Los Angeles, CA, USA).

Quantification of Fibronectin (FN) in serum using enzyme-linked immune-sorbent assay (ELISA) technique

The technique was performed as reference (Attallah *et al.*, 2007) with some modifications as following; serum samples were diluted in coating buffer at 1:50, microtiter wells were blocked with 0.1% blocking buffer, monoclonal antibody specific for FN was diluted at 1:50, and goat anti-mouse IgG conjugate alkaline phosphatase (Sigma) was diluted at 1:300. Absorbance of produced colors were detected on the ELISA reader (Robonik, India) at 490 nm.

Statistical analysis

All statistical tests were performed using SPSS software v.15.0 (SPSS Inc., Chicago, IL) and GraphPad Prism package v.5.0 (GraphPad Software, San Diego, CA). Normally distributed data were expressed as mean± standard deviation (SD), while non-normally distributed data were shown as median± interquartile range (IQR). Significant difference was assessed by one way ANOVA Tukey's post hoc test or Kurskal-Wallis's test as appropriate. $P<0.05$ is considered significant. Area under receiver-operating characteristic (ROC) curve (AUC) was used to assess the diagnostic accuracy of FN in HCC detection. AUC of 1.0 describes the best performance test. Equilibrium between sensitivity and specificity for different values of the test was determined from ROC. The top cut-off value for the performance test was chosen taking specificity, sensitivity, and accuracy into consideration.

Results

Characteristics of patients:

Representative data of all patients are illustrated in (Table 1). This work focused on the detection of HCC patients and differentiating them from patients with cirrhosis (F4 group). Primarily, older age patients were associated with the presence of HCC more than younger agers with mean age ± SD of 57.2±8.9. Moreover, the HCC patient group showed increased levels of some clinical markers such as: AST, total bilirubin, and AFP, otherwise it showed decreased levels of albumin in reference to the F4 group with *p* values <0.0001. Interestingly, basic analysis of the data demonstrated the value of AFP in discriminating HCC patients from cirrhotic patients with *p* value <0.0001.

Table 1: Patients` liver function tests

Variables*	**F1-F3* (n=165)**	**F4** (n=163)**	**HCC*** (n=117)**	***P* value**
Age (years)	42.9±8.6	51.1±9.9	57.2±8.9	<0.0001
Gender				
Male, no (%)	114 (69.1)	111 (68.1)	93 (77.5)	0.080
Female, no (%)	51 (30.9)	52 (31.9)	24 (20.5)	
AST (U/L)	49.0 (36.0-60.0)	50.0 (35.0-72.0)	75.0 (52.0-97.0)	<0.0001
ALT (U/L)	59.1 (44.8-79.0)	38.0 (25.0-67.8)	44.0 (35.0-55.0)	<0.0001
Albumin (g/L)	42.7±3.9	37.8±4.9	32.1±6.3	<0.0001
Bilirubin (mg/dL)	0.7 (0.5-1.0)	0.9 (0.6-1.3)	1.8 (1.2-3.0)	<0.0001
AFP (U/L)	2.7 (1.6-4.5)	5.8 (2.9-12.7)	190.8 (11.9-583.0)	<0.0001

Reference value: aspartate aminotransferase (AST) up to 40 U/L; alanine aminotransferase (ALT) up to 45 U/L; albumin 38-54 g/L; bilirubin up to 1 mg/dL; α-fetoprotein (AFP) up to 10 U/L. Normally distributed data were expressed as mean± standard deviation (SD) while non-normally distributed data were shown as median (interquartile range). Significant difference was assessed. One way ANOVA test or Kurskal-Wallis test as appropriate. $P<0.05$ is considered significant.

*F1-F3 is the fibrotic liver patient group, **F4 is the cirrhotic liver patient group, ***HCC is the hepatocellular carcinoma patient group.

Distribution of fibronectin (FN) levels among patient groups

For assessing serum FN in various patient groups using ELISA, a mono-specific FN antibody was employed as a probe. Patients with HCC exhibited a significantly higher (P =0.0001) concentration of FN than patients with fibrotic liver (F1-F3) and cirrhotic liver (F4), as shown in (Figure1). Furthermore, FN levels among HCC patients revealed 2.1- and 1.7-fold greater than fibrotic liver (F1-F3) and cirrhotic liver (F4) patients, respectively; (figure 1B-1C).

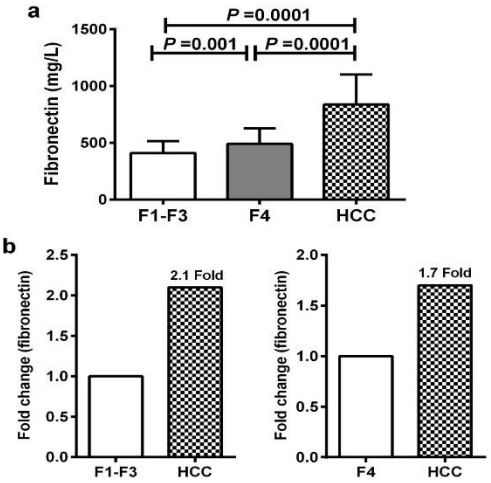

Figure 1. Distribution of fibronectin among different group of patients. (a) Levels of fibronectin in sera of studied patients using ELISA; The distribution of observed fold changes for fibronectin between HCC patients versus those with (b) liver fibrosis (F1-F3) and (c) cirrhosis (F4).

Fibronectin's diagnostic efficacy in the identification of HCC

We then investigated whether FN levels can be used to differentiate between HCC and cirrhotic liver patients. Using the ROC curve analysis, we assessed the diagnostic accuracy of FN in HCC detection. In accordance with the ROC analysis, FN achieved an AUC of 0.95 for separating HCC patients from fibrotic liver patient (F1-F3) group with specificity of 91.5% and sensitivity of 83.7%; (figure 2A). More importantly, ROC analysis of FN produced an AUC of 0.89 for separating HCC patients from cirrhotic liver patient group (F4) with specificity of 80.4% and sensitivity of 83.7%; (figure 2B). Furthermore, ROC curves of FN displayed an AUC of 0.93 with specificity of 86% and sensitivity of 83.7%, this was for separating all non-cancerous patients from HCC; (figure 2C).

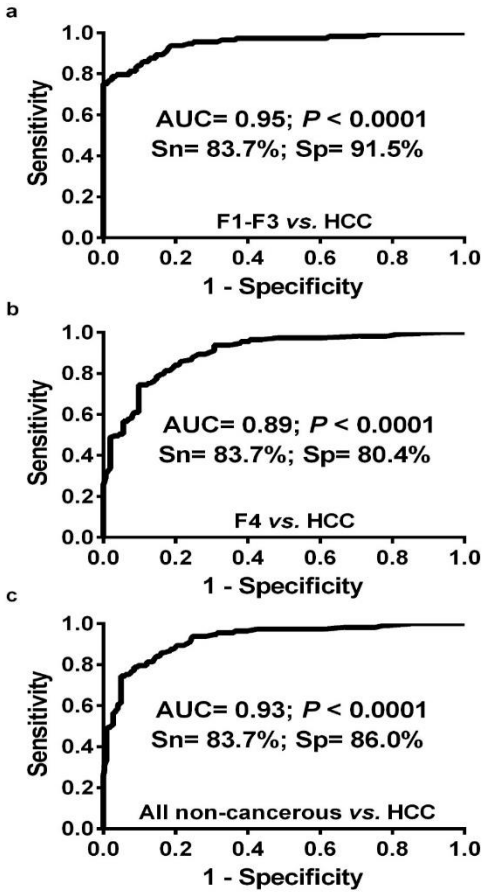

Figure 2. Area under receiver-operating characteristic curve (AUC) of fibronectin for separating patients with HCC from (a): liver fibrosis patients (F1-F3), (b): cirrhotic patients (F4) and (c): all non-cancerous patients. Sn=sensitivity and Sp= specificity.

Distribution of FN level in HCC patients according to histological disease progression

Statistically studied data of elevated levels of FN among HCC patients showed correlation with the progression of some histological features of the tumor, when compared to cirrhotic liver patient group (F4) with p values < 0.05. These features included the existence of vascular invasion, the number and the size of nodules, and Child-Pugh Turcotte score. We observed that increased levels of FN were associated with the presence of vascular invasion, the presence of multiple nodules, increase of tumor size ≥3Cm, and advanced Child-Pugh Turcotte score (Child C); (figure 3A-D).

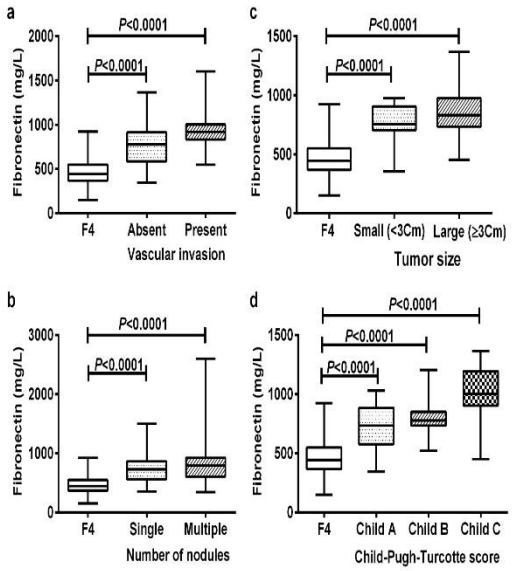

Figure 3. Distribution of fibronectin level in HCC patients according to different histological features including (**a**): vascular invasion, (**b**): number of nodules, (**c**): size of nodules and (**d**): Child-Pugh-Turcotte score. $P<0.05$ is considered significant whereas F4 is the reference group.

Discussion

The viral infection represents a significant threat to the liver, especially HCV, whereas its persistence in liver leads to serious injuries including cirrhosis and HCC (**Mandoh *et al.*, 2021**). End-stage (cirrhotic) patients still face a great danger of developing HCC with risk rates of 2-4%, annually (**Frenette *et al.*, 2019**). Deposition of FN in the ECM was found to be connected to the progression of chronic liver injuries such as cirrhosis and liver cancer (**Roy *et al.*, 2023**). This connection guided the scientists to explore the benefit of the use of some serum biomarkers in assessing the progression of cirrhosis into liver cancer, involving HCC. Hence, our work focused on identifying FN levels among HCV-derived HCC patients and evaluating its interference with progression of the tumor histological features in end-stage patients.

In our study we quantified FN levels in different patients' groups, and it was observed that patients who had HCC were accompanied by a significant increase (P=0.0001) in the concentration of FN when compared to fibrotic (F1-F3) and to cirrhotic patients (F4). Whereas, HCC patients displayed a 2.1-fold and 1.7-fold increase in FN level over fibrotic and cirrhotic patients, respectively. These results agree with the evidence mentioned earlier about FN involvement in the process of tumorigenesis (**Wang *et al.*, 2021**). The promising results encouraged us to further investigate the ability of FN to differentiate between cirrhotic and HCC patient groups. Using ROC analysis, FN yielded an area under curve (AUC) of 0.95 for differentiating HCC patients from fibrotic patient group (F1-F3) with specificity of 91.5% and sensitivity of 83.7%. Moreover, the ROC of FN produced an AUC of 0.93 for separating all non-cancerous patients from HCC patients with specificity of 0.86% and sensitivity of 0.83.7%. Above all, ROC analysis of FN achieved an AUC of 0.89 for separating HCC patients from cirrhotic patient group (F4) with specificity of 80.4% and sensitivity of 83.7%. These results were superior in comparison to the biomarker AFP, which resulted AUCs ranging from 0.73-0.83 in detecting HCC, according to (**Hughes *et al.*, 2021**). Comparing FN with GALAD score; combining different serum biomarkers (AFP, AFP-L3, DCP), that detect HCC with an AUC more than 0.88 (**Johnson *et al.*, 2014**), FN is still more valuable in differentiating cirrhosis and HCC patients. Another biomarker glypican-3 displayed an AUC of 0.78 for differentiating cirrhosis from HCC (**Xu *et al.*, 2019**), which is inferior to our results. Given that genetic diversity of HCC impeded the dependency on certain biomarkers in terms of practical application as diagnosing markers (**Debes *et al.*, 2021**), we attempted to investigate this obstacle by assessing the correlation of FN with the progression of HCC histological features. The data of FN levels in HCC group were statistically analyzed in reference to F4 group and p value < 0.05 were significant. Accordingly, it was observed that the increase in FN levels among HCC patients correlated with the presence of vascular invasion, and the increase in the nodule numbers. Additionally, elevated levels of FN were associated with large tumor size ≥ 3 Cm, and advanced Child-Pugh Turcotte score (Child D).

Conclusion

In conclusion, FN showed promising results in the identification of HCC. Furthermore, notable outcomes were shown for FN participation in the progression of cirrhosis into HCC based on various histological characteristics. This work recommends more observation and

investigation of FN as an efficient single biomarker for HCC detection in early stages of the disease.

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الملخص العربي

عنوان البحث: بصمة الفيبرونكتين في تتبع تطور سرطان الخلايا الكبدية

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يهدف عملنا إلى دراسة استخدام الفيبرونكتين (FN) في تتبع تطور سرطان خلايا الكبد الناتج عن الالتهاب الكبدي الوبائي سي. يعتبر الفيبرونكتين من أهم البروتينات الموجودة حول الخلايا (ECM) والذي يخضع لتغييرات فيزيائية وكيميائية كبيرة أثناء تطور مراحل السرطان. لقد قمنا بقياس مستويات الفيبرونكتين في مصل دم مرضى سرطان خلايا الكبد ومرضى تليف الكبد ومرضى تشمع الكبد. قدمت لنا النتائج دلائل على زيادة تركيز الفيبرونكتين في مصل دم مرضى سرطان خلايا الكبد عن المرضى الآخرين بمقدار ٢,١و١,٧ ضعف على التوالي. كما أظهرت التحاليل الإحصائية قدرة الفيبرونكتين على التحقق من وجود سرطان خلايا الكبد وكذلك قدرته على التفريق بين مرضى السرطان ومرضى التشمع حيث كانت قيمة المساحة تحت منحنى الخصائص (AUC=0.89) بحساسية مقدارها ٨٣,٧٪وتخصصية مقدارها ٨٠,٤٪. علاوة على ذلك، كانت قيمة المساحة تحت منحنى الخصائص (AUC=0.93) مع تخصصية ٨٦٪ وحساسية ٨٣,٧٪، وكانت هذه القيم لقدرة الفيبرونكتين على التمييز بين جميع المرضى غير السرطانيين عن مرضى سرطان خلايا الكبد. كما أوضحت الاحصائيات ارتباط الفيبرونكتين بتطور مراحل سرطان الخلايا الكبدية من حيث السمات النسيجية مثل وجود أوعية دموية مغذية للورم وزيادة عدد العقد السرطانية وكبر حجمها. تسلط النتائج التي توصلنا إليها الضوء بشكل لا لبس فيه على المشاركة الكبيرة للفيبرونكتين في تطور سرطان الخلايا الكبدية.