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Assessment of Fibrosis in Cases with Hepatitis C Infection; a Comparative Study Between Liver Transient Elastography and Liver Biopsy(using Routine and Immunohistochemical Staining)

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Abstract: Hepatitis C virus (HCV) infection and its complications are among the leading public health challenges in the world especially in Egypt. The prognosis and management of chronic liver diseases depend mainly on the amount and progression of liver fibrosis that could be synthesized by the hepatic stellate cells (HSCs). Because of the limitations of liver biopsy, noninvasive alternatives including transient elastography (fibroscan) have been developed. The aim of the work:This study was conducted to compare transient elastography (fibroscan) with histological examination by liver biopsy for the diagnosis of hepatic fibrosis among chronic hepatitis C and to evaluate the role of HSCs in fibrosis using immunostaining in the studied cases.Material and methods: 60 patients were subjected to liver biopsy and fibroscan examination. Liver fibrosis was staged according to the METAVIR system and alpha smooth muscle actin (α-SMA) immunostaining was used to assess the activated hepatic stellate cells (HSCs). The diagnostic performance of fibroscan was assessed by the analysis of the receiver operator characteristics curve (ROC), the sensitivity, the specificity and diagnostic accuracy.Concluding that the major role of transient elastography is the exclusion of cases with bridging fibrosis and cirrhosis. However, it cannot replace biopsy for the diagnosis of significant fibrosis. Hence, transient elastography could be useful in monitoring liver disease and follow up, in cases where liver biopsies are contraindicated. *Immunostaining of* Hepatic stellate cells *by α-SMA* could shed light to diagnosis of early fibrosis for better therapeutic role in prevention of fibrous tissue formation.

Key words: Hepatitis C · Elastography · Liver Biopsy · Fibrosis · Hepatic Stellate Cells

INTRODUCTION

The stage of liver fibrosis in patients with hepatitis C viral infection (HCV) is the most important predictor of disease progression that determines the need for antiviral therapy [1]. Liver fibrosis originally has been thought to be as a one-way street, now is recognized as a dynamic process with potential for significant resolution [2]. In early stage of HCV infection, the immune system generates antibodies to eradicate the virus, the hepatocytes are damaged once the infection becomes chronic through direct cellular toxicity and local stimulation of inflammatory cytokine expression, this activates hepatic stellate cells (HSCs) that on activation lose their cytoplasmic lipid droplets, forming multiple microfilaments that consist mainly of alpha-smooth muscle actin (α-SMA); triggering liver fibrosis [3, 4], these cells synthesize collagen types III, IV and in small quantities type I, mediated through a series of signaling molecules released by inflammatory cells, damaged hepatocytes, Kupffer cells and sinusoidal endothelial cells. The proliferation of HSCs results in; increase in type I collagen synthesis and extracellular matrix (ECM) accumulation, with a reduction in its degradation [4]. The first percutaneous liver biopsy (LB) was performed in 1923 and in the past 50 years, it has become the primary tool for diagnosing and staging of liver disease. However, liver biopsy is an invasive and painful procedure it

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sometimes carries a risk of life-threatening complications [5]. An obvious trend in clinical practice observed in the latter years consists of finding a correct method for liver fibrosis evaluation in a noninvasive way, both by biochemical tests as well as imaging methods, as an alternative to LB [6]. The imaging methods have the advantage of being noninvasive and, at the same time, they allow a complete evaluation of the entire organ with a precise assessment of the disease severity when fibrosis does not affect the liver uniformly, unfortunately these methods have been limited for the tracing of cirrhosis and its complications [7]. The predominant role for radiological imaging is the confirmation of cirrhosis in patients with high clinical suspicion of advanced chronic liver diseases. Furthermore, radiological determinants of cirrhosis are complementary in cases where the biopsy results are intermediate or at variance with the clinical impression [8]. Transient hepatic elastography is a non-invasive mean determining fibrosis[9],which could be sensitive for staging hepatic fibrosis, this rapid noninvasive technique reproducibly measures the mean hepatic tissue stiffness [10, 11].

The aim of the work is to assess liver fibrosis by measuring liver stiffness using transient elastography (fibroscan) in patient with chronic hepatitis C in comparison with that of routine liver biopsy and to evaluate the role of HSCs in fibrosis using Alpha smooth muscle actin.

MATERIALS AND METHODS

Sixty chronic hepatitis cases from National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt. HCV infection was confirmed by the presence of viremia using PCR. The patient's ages ranged between 24 and 59y. They included 35 male and 25 female. All the patients were subjected to the followings: careful history taking and full clinical examinations, laboratory investigations as complete blood count (CBC), liver profile (serum albumin, bilirubin, ALT, AST, prothromin time, prothromin concentration), kidney function (urea and creatinine), hepatitis markers (HbsAg, HCV- Ag) using third generation ELISA test, abdominal ultrasound and quantitative HCV RNA by PCR.

Liver Biopsies: Were obtained; sections were examined by H&E stain and by Masson trichrome (MT) stain for routine histopathological diagnosis and assessment of fibrosis, liver biopsy specimens were analyzed into F0, F1, F2, F3 and F4 according to the METAVIR scoring system.

Immunohistochemical Study: Sections were prepared for immunostaining and subjected to monoclonal antibodies for α-SMA. Results were evaluated semi-quantitatively by two independent pathologists based on the staining determined by the percentage of positive cells on an x100 magnification in nearly 5 areas. The α-SMA-positive vascular smooth muscle cells were excluded from scoring (considered as internal control). Staining up to 3% was considered negative, score (1) or mild staining 3-33%, (2); moderate staining : 34-66%, (3) strong attaining if more than 66% of mesenchymal cells in the portal tracts and fibrous septa [12].

Liver Transient Elastography (Fibroscan): All patients were studied using the non-invasive method of transient elastography (Fibroscan, Echosens, Paris, France). We used both ultrasounds of 5 MHz and low frequency elastic waves. The system consisted of a probe with an ultrasonic transducer mounted on the axis of a vibrator. This vibrator induced a wave of mild amplitude and low frequency to the tissue. Thus, an elastic shear wave was created that propagates in the tissue and in the meantime a pulse-echo ultrasound is performed to follow the shear wave and measures its velocity. The velocity of propagation was directly related to the tissue stiffness. The harder the tissue, the faster the shear waves propagated. Measurements were totally non-invasive and performed on the right lobe of the liver through intercostals spaces between 25 and 45 mm from the skin surface. For each patient, the obtained elasticity value was the median of several measurements (usually 10) and the results were expressed in kilopascals (kPa). Transient elastography measured the liver stiffness in a volume that is approximated a cylinder 1 cm wide and 6 cm long. This volume was at least 100 times bigger than a biopsy sample and therefore is far more representative of the hepatic parenchyma. The technique was performed by the same blinded gastro-enterologist and at least 10 validated measurements were carried out in each patient one day prior to liver biopsy. Measurements were performed on the right lobe of the liver through the intercostals spaces on patients lying in the dorsal decubitus position with the right arm in maximal abduction. The median value (expressed as kilopascals, kpa) was kept as representative of the liver elastic modulus and staged as F1(up to7 kpa), F2(7-11 kpa), F3(14.5 up to 17.5 kpa), F4(≥ 17.5- 75kpa) [13].

Statistical Analysis: Data were collected; coded and analyzed using SPSS Software Version 15 under Widows Vista, Descriptive analysis was performed followed by

inferential statistics. Test of Significance used included: Chi- Square test and ANOVA (Analysis of Variance). P < 0.05 = Significant and P < 0.01 = highly significant, corrected by Kruskal-Wallis Test whenever needed.

The diagnostic performance of fibroscan was assessed by the analysis of the receiver operator characteristics curve (ROC), the sensitivity, the specificity and diagnostic accuracy.

RESULTS

Among all the studied patients; 35of them were males (58.3% of cases) and 25 were females (41.7% of cases) the age ranged between 24 and 59 years with mean age 44.95±10.04 SD. Of the all studied patients; according to Metavir score, liver biopsies (stained by H&E, MT) showed that9 patients were scored as F0 fibrosis (15 %), twelve patients were F1 (20 %), 18(30%) of our patients were F2, 14 cases (23.3%) were F3 and only 7 patients (11.7%) were scored as F4. Liver stiffness values ranged from 2.90 to 75 kPa (median12.10 kPa). The degree of fibrosis by fibroscan and liver biopsy in the studied cases was tabulated in Table 1.

In this work there was discrepancy in the detection of liver fibrosis between fibroscan and Metavir scoring by LB as we found nine patients (15%) with F0 on LB, but none of the cases were categorized as F0 by fibroscan, twelve patients (20%) showed F1 on LB, the same number of cases was categorized as F1 by fibroscan, 18 (30%) patients showed F2 on LB, while by fibroscan 23 cases (38.3%) were F2, 14 cases(23%) were F3 on LB, higher by fibroscan 19 cases(31.7%) were of F3 category. Seven patients (11.7%)showed F4 on LB, nearly similar by fibroscan 6 cases showed F4(10%).For further clarification of our results, we tried to classify our patients into non significant fibrosis; fibrosis stages (F0, F1) and significant fibrosis; fibrosis stages (F2 or F3) and advanced fibrosis (cirrhosis) F4. We found twenty one patients (35%) showed no significant fibrosis (F0, F1) when diagnosed by liver biopsy, only twelve patients (20%) by fibroscan. Thirty nine cases by liver biopsy have advanced fibrosis while by fibroscan 48 cases have advanced fibrosis. Approximately the same number of cases diagnosed as cirrhosis (7 by liver biopsy and 6 by fibroscan). Table 2, Figures 1; a,b,c show ROC curves of the diagnostic accuracy of fibroscan for staging fibrosis compared with liver biopsy. The AUROCs for significant fibrosis (F2 or greater), bridging fibrosis or cirrhosis (F3 or greater) and cirrhosis (F4) were 0.74 (95% CI 0.68 to 0.80), 0.86 (95% CI 0.84 to 0.91) and 0.94 (95% CI 0.90 to 0.97), respectively.

The diagnostic accuracy of fibroscan confirmed the excellent diagnostic accuracy of fibroscan, in identification of advanced fibrosis and cirrhosis.

Table 1: Degree of fibrosis by fibroscan and liver biopsy (H&E, MT stains) in the studied group:

Variables	Number	Percent
Liver biopsy*		
F0	9	15
F1	12	20
F2	18	30
F3	14	23.3
F4	7	11.7
Fibroscan* F0	0	00.0
F1	12	20
F2	23	38.4
F3	19	31.6
F4	6	10

Table 2: Specificity and sensitivity of Metavir scores and fibroscan (kpa)

	≥ F2	≥F3	F4
Fibroscan kpa cut-off	7.85	8.15	12.7
Sensitivity(%)	87.4%	95.2%	95.7%
Specificity%	66.7%	46.2%	94.3%
Area under the curve (AUC)	0.76	.88	.991
DA (95% CI)	.68 to .80	.84 to .91	.90 to .97

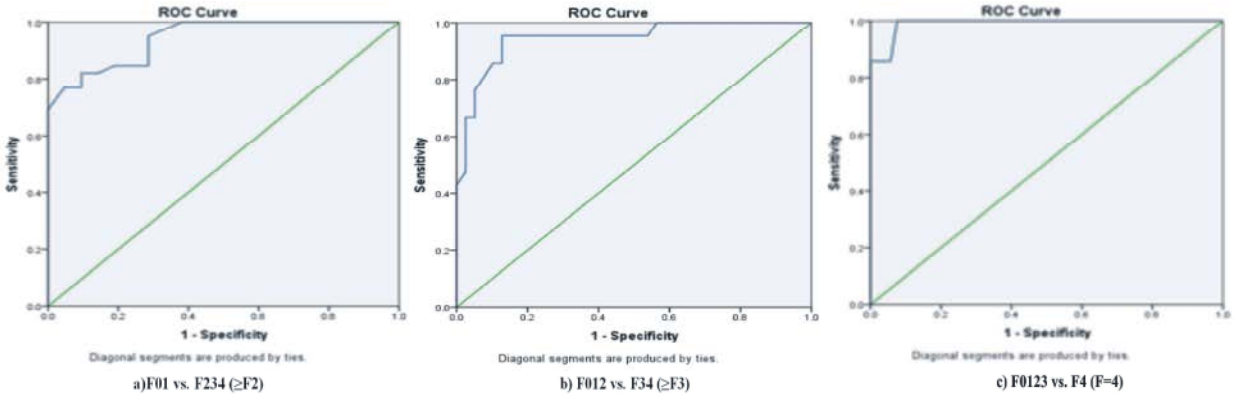

Fig. 1: Cut off values for fibroscan and Metavir scoring in diagnosis of fibrosis (ROC curves a,b,c).

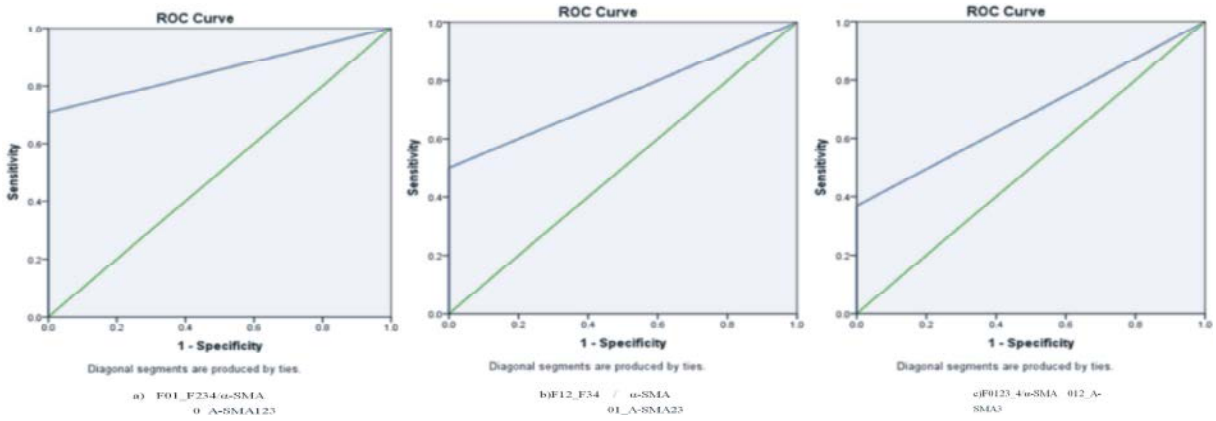

Fig. 2: Cut off values for Metavir scoring and α-SMA immunostaining in diagnosis of fibrosis (ROC curves).

Table 3: Cross tabulation of α-SMA immunostaining and fibrosis scoring by Metavir

			Fibrosis Metavir score					
			F0	F1	F2	F3	F4	Total
α-SMA immunostaining	0	Count	5	0	0	0	0	5
		% within fibrosis metavir score (biopsy)	55.6%	0.0%	0.0%	0.0%	0.0%	8.3%
	1	Count	4	9	0	0	0	13
		% within fibrosis metavir score (biopsy)	44.4%	75.0%	0.0%	0.0%	0.0%	21.7%
	2	Count	0	3	13	7	0	23
		% within fibrosis metavir score (biopsy)	0.0%	25.0%	72.2%	50.0%	0.0%	38.3%
	3	Count	0	0	5	7	7	19
		% within fibrosis metavir score (biopsy)	0.0%	0.0%	27.8%	50.0%	100.0%	31.7%
Total		Count	9	12	18	14	7	60
	% within fibrosis metavir score (biopsy)		100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Chi-square(p value)=0.000

Table 4: Sensitivity and specificity of Metavir score and α-SMA immunostaining score

	F01_F234 vsα-SMA0_A-SMA123	F012_F34 vs.α-SMA 01_A-SMA23	F0123_F4 vs.α-SMA 012_A-SMA3
Sensitivity (%)	70.9%	50%	36.8%
Specificity%	100	100	100
Area under the curve (AUC)	.855	.750	.684
95% CI	0.748 to 0.961	0.630 to 0.870	0.523 to 0.845

Studying the role of HSCs in fibrosis; we found that these cells were early activated before fibrous formation as we were unable to detect fibrosis by liver biopsy stained by H&E or MT stains in 9 cases (15%).On the other hand,four of them showed positive α-SMA staining and five were negative, 13 cases (21.7%) showed score 1 α-SMA$^+$ HSCs, α-SMA $^+$HSCs were seen in 23 cases (38.3%) were scored 2 and 19 cases (31.7%) were scored 3 (Table3) .The utilization of immunostaining scoring of α-SMA to predict early fibrosis; fibrosis stages (F0, F1) and their role in significant fibrosis; fibrosis stages (F2, F3, F4) and cirrhosis (F4) were illustrated in Table 4. The *AUROC curve* of α-SMA for significant fibrosis (F2–F4), advanced fibrosis (F3–F4) and cirrhosis (F4) were o.855;0.750and 0.684 respectively. These results confirmed the excellent diagnostic accuracy of α-SMA, in particular for the identification of early formation of fibrosis Figure 2(a,b,c), Figure 3(a,b,c,d,e,f,) illustrated the detection of fibrosis by fibroscan, routine biopsy and HSCs immunostaining in early and late cases of fibrosis.

These results confirmed the excellent diagnostic accuracy of α-SMA, in particular for the identification of minimal fibrosis.

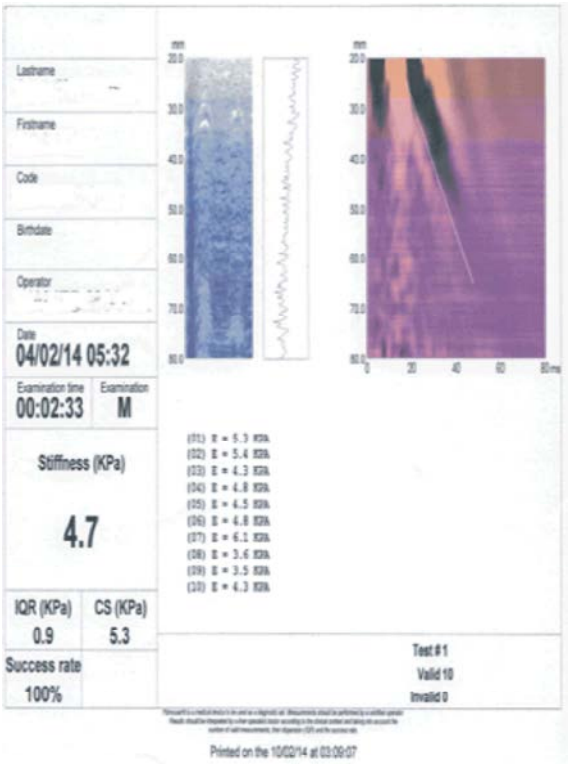

Fig. 3a: A photograph of fibroscan score 0 for a case diagnosed as F0 by LB on routine stains.

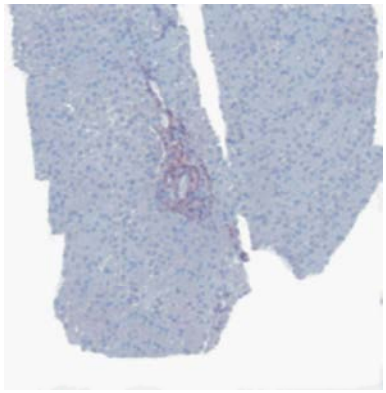

Fig. 3b: Score1 for α-SMA immunostaining of previous case

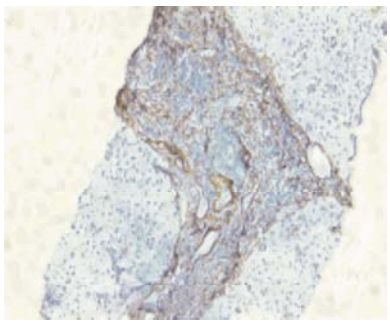

Fig. 3c: Score 2 immunostaining

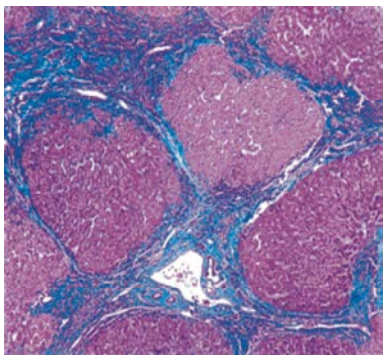

Fig. 3d: A case of liver cirrhosis MT stain Metavir score F4.

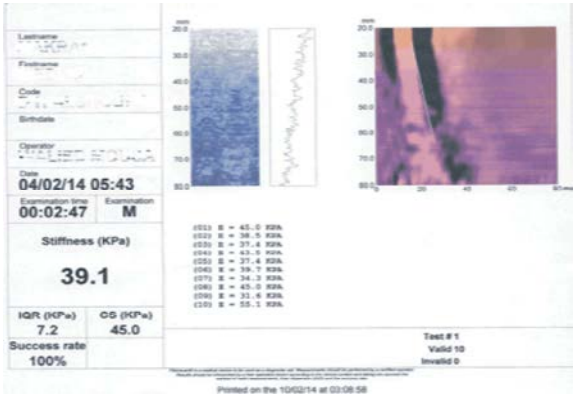

Fig. 3e: The fibroscan of previous case showing fibrosis scoreF4.

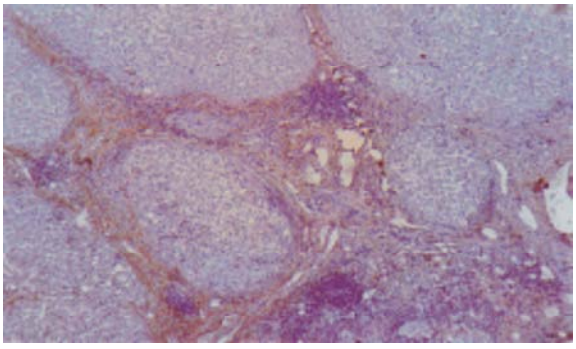

Fig. 3f: Score 3 of α-SMA immunostaining in previous case of chronic hepatitis C Metavir score F4.

DISCUSSION

In Egypt; Egyptian Demographic Health Survey (EDHS), a cross sectional survey including hepatitis C virus (HCV) biomarkers, was conducted in 2008 on a large nationally representative sample, the HCV prevalence among the 15–59 years age group to be estimatedas14.7%. Accordingly, as Egypt has the highest HCV prevalence in the world, this unparalleled level of exposure to this

infection appeared to reflect a national level epidemic. It has been postulated that schistosomiasis is a chronic and debilitating disease in Egyptian and remains one of the most prevalent parasitic infections, hepatitis C epidemic is caused by extensive iatrogenic transmission during the era of parenteral-anti schistosomal-therapy (PAT) mass-treatment campaigns. Today, HCV infection and its complications are among the leading public health challenges in Egypt [14, 15]. Liver biopsy (LB) has always been represented as the standard of reference for assessment of hepatic fibrosis [16]. Although biopsy is used to stage most cases of liver disease, it is well known that this procedure has several limitations [17] and hence development of non-invasive assessment methods to quantify hepatic fibrosis is justifiable. But, any non invasive method needs to be compared with the standard LB [18]. This study is to compare accuracy of the non-invasive examination of hepatic stiffness by (fibroscan) in comparison to the standard LB for assessment of hepatic fibrosis in 60 adult Egyptian chronic HCV patients. The diagnostic performance of fibroscan was assessed using sensitivity (Se), specificity (Sp) and receiver operating characteristic(ROC) curves. The ROC curve is a plot of sensitivity versus 1-specificity for all possible cut-off values. The most commonly used index of accuracy is the area under the ROC curve (AUROC), with values close to 1.0 indicating higher diagnostic accuracy.

Our data showed that 58.3% of our randomly chosen samples were males. This is in agreement with general epidemiological characteristics of HCV infection in Egypt, which might be explained by the high prevalence of schistosomiasis infection and anti schistosoma treatment with intravenous drugs that was higher between males more than females [19]. In the present study we found discrepancies on studying the effectiveness of transient elastography (fibroscan) as a method for diagnosis of hepatic fibrosis compared to that of liver biopsies stained by H&E, MT. Evaluating insignificant fibrosis (stages F0, F1) and significant fibrosis; (fibrosis stages F2, F3, F4) we found 21 patients (35%) showed no significant fibrosis (F0, F1) when diagnosed by liver biopsy and twelve patients (20%) by fibroscan as a method of diagnosis: In this work, there was discrepancy in the detection of liver fibrosis between fibroscan and LB as we found nine patients (15%) with F0 on liver biopsy, but none of the cases were categorized as F0 by fibroscan, twelve patients (20%) showed F1 on liver biopsy, the same number of cases was categorized as F1 by fibroscan, 18 (30%) patients showed F2 on liver biopsy, while by fibroscan 23 cases(38%) were F2, 14 cases(23%) were F3 on liver biopsy, while higher number by fibroscan 19 cases(31.7%) were of F3 category. Seven patients (11.7%) showed F4 on liver biopsy, nearly similar by fibroscan 6 cases showed F4(10%). Liver stiffness values by fibroscan ranged from 2.90 to 75 kPa. The areas under ROC curve for the diagnosis of fibrosis F=2, F=3 and F=4 were 0.763, 0.883 and 0.991, for the cut-off values of 7.85 kPa, 8.15 kPa and 12.7 kPa. These results suggest that fibroscan performs well in identifying severe fibrosis or cirrhosis, but is less accurate in identifying lesser degrees of fibrosis. This is important because F2 is a threshold for initiating treatment [19]. In agreement with this, different studies [20-23] present AUROC (area under the receiver operating characteristic curve) values, a commonly used index of diagnostic accuracy where values close to 1.0 represent high diagnostic accuracy. In terms of fibroscan's ability to discriminate degrees of fibrosis, as staged on the Metavir scale (F0 to F4 where F0=no fibrosis and F4=cirrhosis), the AUROC ranges across the studies were F>2, 0.72 to 0.88; F>3, 0.90 to 0.91; and, F=4, 0.95 to 0.99. Colletta et al. performed a study on 2005 to evaluate the diagnostic performance of fibroscan in patients with normal liver enzymes and they found that for identifying patients with significant fibrosis (Metavir F2 or F3) sensitivity, specificity, negative and positive predictive value were all 100% [23]. This finding has sparked debate [20].

Although activated HSCs is immunohistochemically stained with a series of antibodies, α-SMA represents a trustworthy marker in emphasizing their filaments. HCV replication generates a series of factors that intervene in modulating HSCs synthesis, by increasing type I and III procollagen expression, as well as by inhibiting the fibrinolytic activity of matrix metalloproteinase MMPs [4]. By studying the role of HSCs in the present study we noticed that the modulated α-SMA + HSCs might predict early fibrosis as 21 patients (35%) showed no significant fibrosis (F0, F1) when diagnosed by liver biopsy, four of which were positive while 5 patients of which were negative for α-SMA. (Sensitivity of α-SMA 70.9%, Specificity of α-SMA 100 %). While for the utilization of α-SMA to differentiate between non cirrhotic liver; fibrosis stages (F0, F1, F2 and F3) from cirrhotic liver; fibrosis stage (F4) we regrouped the patients into two groups with one group including non-cirrhotic patients (F0, F1, F2 and F3). 19 cases (31.7%) were score 3 for α-SMA, only 7 patients showed cirrhosis (F4) when diagnosed by liver biopsy using routine H&E and MT stains. These results confirmed the excellent diagnostic accuracy of α-SMA, in particular for the identification of early formation of fibrosis (Sensitivity 36.8%, Specificity 100).

CONCLUSION

Hepatic stellate cells may have an important role in initiation of fibrosis and could detect very early fibrosis. This could shed light to immunostaining role in the diagnosis of early fibrosis for better therapeutic role in prevention of fibrous tissue formation, liver biopsy might be still an accurate method for diagnosis liver scoring of fibrosis. As transient elastography is an easy and quick clinical non-invasive method to perform, results are available immediately; this technique might predict significant fibrosis in late stages. Hence, transient elastography could be useful in monitoring liver disease and follow up, which might be used in cases where liver biopsies are contraindicated.

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