Serum hyaluronic acid level does not reliably differentiate minimal and significant liver disease in chronic hepatitis C

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ABSTRACT

الأهداف: تقييم القيمة التنبؤية لحمض الهيالورونيك من أجل تقييم حالة تليف والتهاب الكبد لدى مرضى التهاب الكبد ج المزمن.

الطريقة: أُجريت هذه الدراسة المقطعية في جامعة زيادين، كراتشي، باكستان وذلك خلال الفترة من يونيو 2006م إلى يوليو 2010م. شملت الدراسة 98 مريض مصاب بالتهاب الكبد ج المزمن، حيث كان عدد الذكور 52 مريض (53%)، وعدد الإناث 46 مريضة (47%)، فيما تراوحت أعمار المشاركين في الدراسة من 20–60 عاماً (36.0±10.5). لقد تم تقييم تليف الكبد على أساس مقياس مكون من 5 درجات ويتراوح ما بين F0 إلى F4، فيما قُيم الالتهاب على أساس مقياس مكون من 4 درجات يتراوح ما بين A0 إلى A3. ولقد تم تقسيم المرضى إلى مجموعات المرضى المصابة بدرجة متدنية من المرض (F<2, A<2)، ومجموعات المرضى المصابة بدرجة شديدة من المرض (F≥2, A≥2). وتم قياس حمض الهيالورونيك في مصل الدم باستخدام اختبار الإليزا، فيما حُللت قيمة التشخيص بواسطة منحنى روك (منحنى خصائص تشغيل المستقبل).

النتائج: أشارت نتائج الدراسة إلى ظهور مرض الكبد لدى 46 مريض (47%)، وقد كانت قيم حمض الهيالورونيك متباينة بشكل واضح بين المجموعات المصابة بدرجات شديدة من المرض (p=0.001). بلغت قيمة المنطقة الموجودة تحت منحنى روك لمجمل المرض 0.716. ولقد ظلت القيمة التنبؤية السلبية لمجمل المرض 71% وذلك بانخفاض مستويات حمض الهيالورونيك بمقدار 20 نانوغرام/مل. في حين تم الحصول على القيمة التنبؤية الموجبة 85% عند 60 نانوغرام/مل، والقيمة التنبؤية الموجبة 100% عند 120 نانوغرام/مل. وظهرت المستويات العالية من هذه القيم عند 15% و10% من المرضى.

خاتمة: أظهرت المستويات العالية من حمض الهيالورونيك نتائج منخفضة من القيمة التنبؤية السالبة لأمراض الكبد الشديدة. فيما تم الحصول على نتائج مقبولة من القيمة التنبؤية الموجبة لدى مجموعة صغيرة من عينة الدراسة. وهكذا فإن الاعتماد على اختبار مستويات حمض الهيالورونيك قد لا يكون دقيقاً أثناء وضع خطة العلاج.

Objectives: To evaluate the predictive value of hyaluronic acid (HA) for the assessment of liver fibrosis and inflammation in chronic hepatitis C (CHC).

Methods: This cross-sectional study was conducted at Ziauddin University, Karachi, Pakistan from June 2006 to July 2010. Ninety-eight CHC patients, 52 (53%) males, and 46 (47%) females, with an age range of 20-60 years (mean 36.0±10.5) were recruited. Liver fibrosis was staged on a 5-point scale, F0 to F4, and inflammation was graded on a 4-point scale, A0 to A3. Patients were divided into minimal (F<2 and A<2) and significant (F≥2 or A≥2) overall disease groups. The HA was measured in the serum by ELISA. Diagnostic value was assessed through receiver operating characteristic (ROC) curve.

Results: Significant liver disease was present in 46 (47%) patients. Mean serum HA was significantly different among severity groups (p=0.001). Area under ROC curve for overall disease was 0.716. Negative predictive value (NPV) for significant overall disease remained 71% at a low HA level of 20 ng/mL. Positive predictive value (PPV) of 85% was obtained at 60 ng/mL and 100% at 120 ng/mL. Those high levels were present in 15% and 10% of the patients.

Conclusions: Serum HA levels showed a low NPV for significant liver disease. An acceptable PPV was found only in a small proportion of the patients. Hyaluronic acid may not be regarded as a reliable marker for making treatment decisions.

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Around 170-200 million individuals have chronic hepatitis C (CHC) viral infection worldwide, with 3-4 million new infections occurring each year.[1] It poses a major disease burden in the form of liver fibrosis with its complications; cirrhosis, and hepatocellular carcinoma (HCC).[2] The rate of fibrosis progression is slow and unpredictable with cirrhosis occurring in approximately 10-20% over a period of 20 years.[3] Furthermore, in CHC patients, HCC invariably follows cirrhosis, developing in 1-4% of patients per year.[4] Treatment of CHC is difficult to tolerate, costly, and ineffective in approximately half of the patients.[5,6] A high prevalence of the disease, variable natural history, and complex treatment warrants need to identify the patients who need urgent therapy. Treatment is indicated for the patients with F2-4 fibrosis and A2-3 inflammation.[7] Liver biopsy, the current gold standard for the estimation of liver histology, is an invasive procedure associated with complications and has limitations of sampling and inter-observer variability.[8] For the last many years, several noninvasive methods for the prediction of liver fibrosis have been proposed and evaluated in different clinical studies. These include clinical features, serum biochemical assays, and imaging techniques. Among biochemical markers, hyaluronic acid (HA) has extensively been studied especially in CHC.[9] It is a nonsulfated glycosaminoglycan and is a major component of the extracellular matrix (ECM) in all tissues of the body. In the liver, it is mostly synthesized by the stellate cells, increases during fibrogenesis and is released in systemic circulation during remodeling.[10] Serum levels are found to correlate with fibrosis stage and inflammatory grade on liver biopsy.[11] Keeping in view the association of HA to inflammation and fibrogenesis, the aim of the study was to evaluate its predictive value to assess severity of liver fibrosis and inflammatory activity in CHC.

Methods. This cross-sectional study was conducted at Ziauddin University, Karachi, Pakistan during the period June 2006 to July 2010. The patients were selected from the Ziauddin University Hospital and outpatient clinic of the Pakistan Medical Research Council, Research Center, Jinnah Postgraduate Medical Centre, Karachi, Pakistan. The study was carried out in accordance with the principles of Helsinki Declaration and was approved by the Ethics Review Committee of the university. A written informed consent was obtained from each participant. Consecutive, treatment naïve, chronic hepatitis C patients, who underwent liver biopsy were enrolled in the study. Liver biopsy specimens of at least 10 mm length were included. Patients with hepatitis B co-infection, diabetes mellitus, history of alcohol intake, and chronic inflammatory conditions such as rheumatoid arthritis and blood disorders requiring frequent blood transfusions were excluded from the study. Liver biopsy specimens with less than 5 portal tracts or showing pathologies other than chronic hepatitis were also excluded. A percutaneous liver biopsy was performed with 16-18 gauge modified Menghini aspiration needle (Surecut, TSK Laboratories, Tokyo, Japan). Prior to biopsy, a blood specimen was collected for measurement of serum HA. Biopsy specimens were processed for light microscopic examination. Histological features of the liver biopsy specimens were analyzed according to the METAVIR group scoring system,[12] by one pathologist who was unaware of patients' clinical data or serum HA levels. Every specimen was staged for fibrosis on a 5-point scale, F0: no fibrosis, F1: portal fibrosis without septa, F2: portal fibrosis with rare septa, F3: numerous septa without cirrhosis, and F4: cirrhosis. F0 or F1 were classified as minimal fibrosis and F2 to F4 as significant fibrosis. Necroinflammatory activity was graded on a 4-point scale, A0: no histological activity, A1: mild activity, A2: moderate activity, and A3: severe activity. A0 or A1 were classified as minimal activity and A2 or A3 as significant activity. Necroinflammatory lesions were graded through an algorithm, based on focal lobular inflammation/necrosis and piecemeal necrosis. Portal inflammation was not included in the grading algorithm, but was recorded as a prerequisite for the diagnosis of chronic hepatitis. Histological lesions were divided in 2 overall severity groups, minimal disease (F<2 and A<2) and significant disease (F≥2 or A≥2). Furthermore, F3 and F4 were lumped separately as widespread fibrosis.[12] Hyaluronic acid was measured by an enzyme-linked binding protein microplate assay (Corgenix® Inc. Broomfield, Colorado, CO, USA). In this assay, HA binding protein (HABP) is used as a capture molecule.[13] Serum was incubated in HABP coated microwells according to the manufacturer's instructions. After washing, to remove the unbound serum molecules, HABP conjugated with horseradish peroxidase was added to the microwell to form complexes with bound HA. A chromogenic substrate was then added to develop a colored reaction. The intensity of the developed color was measured at 450 nm.

Statistical analysis. The data were entered into the computer and statistical analysis was performed using the Statistical Package for Social Sciences version 17.0

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(SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD) for quantitative variables and frequency and percentages for qualitative variables. An independent t-test was used to compare the quantitative variables. A p-value of less than 0.05 was considered significant. The correlation between serum HA levels and different histological categories was calculated by Spearman's correlation coefficient (rs).

The primary outcome for statistical analysis was evaluation of HA for identification of the patients with minimal and significant overall disease on liver biopsy. Diagnostic value of HA was assessed through receiver operating characteristics (ROC) curve analysis. Area under ROC (AUROC) was calculated for all histological categories. Furthermore, sensitivity, specificity, and positive and negative predictive values were calculated at various cutoff points for overall disease.

Results. Initially, 103 patients were enrolled in the study. Five patients were excluded because biopsy specimen in 4 patients had less than 5 portal tracts, and one biopsy showed a granuloma. Finally, 98 patients, 52 (53%) males and 46 (46.93%) females with an age range of 20-60 years (mean age 36.0±10.5) were selected. **Table 1** summarizes baseline demographic and histological features of the patients. The mean serum HA level was 59.6±109.7 ng/mL. **Table 2** summarizes the distribution of patients in histological categories with corresponding HA levels. **Table 3** also shows the mean serum HA levels in different histological groups. These were significantly different for minimal and significant groups of fibrosis, activity, and overall disease categories (p=0.001 in all). However, this was not significant for widespread fibrosis (p=0.054). A modest linear correlation was found between serum HA levels and fibrosis (rs=0.37), activity (rs=0.54), overall disease (rs=0.34), and widespread fibrosis (rs=0.40) categories. The ROC curve analysis for the diagnostic value of HA level, for different histological categories, is shown in **Table 3**. The best diagnostic value of HA was obtained for significant inflammatory activity. For significant fibrosis and overall significant disease, the diagnostic value remained similar. The HA was found to be least informative for the widespread fibrosis category. The ROC curve for overall disease is shown in **Figure 1**. The diagnostic performance was further analyzed based on the overall disease category. Specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) at different cutoff levels is shown in **Table 4**. The predictive value of HA to exclude the presence of significant disease was 71% at a low level of 20 ng/mL. The PPV for overall significant disease was 85% and 100% at a cutoff level of 60 ng/mL and 120 ng/mL. Fifteen patients out of 98 had a value of 60 ng/mL or more, while only 10 had an HA level of 120 ng/mL or more.

Table 1 - Baseline characteristics of 98 patients.

Characteristic	Value
Age (years) (mean±SD)	36.0±10.6
Male (%)	52 (53)
Hyaluronic acid (ng/mL) (mean±SD)	59.6±109.7
Activity grade (%)	
Absent (A0)	32 (33)
Mild (A1)	40 (41)
Moderate (A2)	20 (20)
Severe (A3)	6 (6)
Fibrosis stage (%)	
No fibrosis (F0)	21 (20)
Portal fibrosis without septa (F1)	35 (36)
Portal fibrosis with rare septa (F2)	29 (30)
Numerous septa without cirrhosis (F3)	10 (10)
Cirrhosis (F4)	3 (3)

Table 2 - Distribution of patients in histological categories with corresponding hyaluronic acid (HA) levels.

Category	n (%)	HA, mean ± SD	*P*-value
*Fibrosis (stage)**			0.001
Minimal	56 (57)	24.4±16.6	
Significant	42 (43)	106.4±155.5	
Activity (grade)†			0.001
Minimal	72 (73)	24.9±17.7	
Significant	26 (27)	155.7±181	
Overall disease‡			0.001
Minimal	52 (53)	23.9±16.4	
Significant	46 (47)	100±150	
Widespread fibrosis§			0.053
Absent	85 (87)	42.5±71.2	
Present	13 (13)	171.4±215.1	

*Minimal = <F2 (significant),
†Minimal = <A2 (significant), A2 and above,
‡Minimal = <A2F2; Significant, ≥A2 or F2,
§Absent = <F3; Present, F3 and F4
F2-F4 = fibrosis, A2-A3 = activity/inflammation

Table 3 - Diagnostic value of hyaluronic acid for histological categories.

Category	AUROC	95% confidence interval
Overall significant disease	0.716	0.611-0.820
Significant fibrosis	0.718	0.610-0.825
Significant inflammatory activity	0.841	0.748-0.934
Widespread fibrosis	0.688	0.510-0.866

AUROC - area under receiver operating characteristics curve

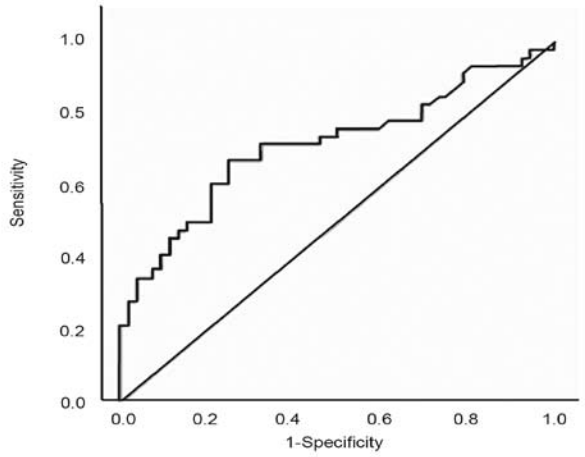

Figure 1 - Receiver operating characteristics curve showing the sensitivity and specificity of hyaluronic acid for the prediction of clinically significant liver disease on biopsy.

Table 4 - Diagnostic value of hyaluronic acid (HA) at different cutoff levels for overall disease.

HA (ng/mL)	Sensitivity	Specificity	PPV*	NPV*
20	74	52	56	71
40	41	90	77	65
60	28	96	85	62
120	22	100	100	61

PPV - positive predictive value, NPV - negative predictive value
*PPV and NPV were calculated for a prevalence of 45%.
All values are expressed in percentage

Discussion. Chronic hepatitis C is one of the leading health problems all over the world.[1] Morbidity and mortality mainly results from progressive liver fibrosis. It is a slow process with marked variability of rate from person to person. Because, only a minority suffers long-term complications, knowledge of the liver histology is not only important for prognostication, it is critical in making treatment decisions, in an individual patient. Keeping in view, the complexities associated with treatment, it seems unlikely that treatment can be offered to every patient. Thus, there seems a need to identify the patients that can safely be deferred for urgent treatment. Use of liver biopsy, the gold standard for the assessment of liver histology, has a few limitations. It is an invasive procedure associated with complications. In addition, diagnostic yield of liver biopsy is affected by sampling errors and inter and intra observer variations. Among non-invasive markers of liver histology, HA has a pathophysiological rationale. It is directly related to the metabolism of ECM, which not only reflects rate of deposition and removal, but also the remodeling of established ECM. Thus, serum levels of HA may reflect both the activity of the process and amount of the ECM undergoing remodeling. Thus, it has a potential to be informative not only for stage of fibrosis, but also for rate of its progression with a more relevant prognostic value.[14]

A major limitation of ECM related markers is that they are not routinely available in hospital settings for clinical use and are not cost-effective. Hyaluronic acid, however, is easily measurable in routine laboratories, both at secondary and tertiary health care levels even in developing countries. In the present study, serum HA levels showed a modest linear correlation with all histological categories. Mean HA levels were also significantly different for different severity groups within all histological categories (p=0.001), except widespread fibrosis (p=0.053). These results are consistent with previous studies showing association of serum HA levels with increasing fibrosis stage and inflammatory grades in CHC and other etiologies.[11,15,16] We could not find any association of widespread fibrosis, possibly because of the smaller number of patients[13] in this category, in our cohort. Anyhow, this was not the primary outcome variable in this study. The ROC curve analysis showed that area under the curve for the diagnosis of significant overall disease was 0.716. Predictive value to exclude the presence of significant disease remained low, being 71% at a low cutoff level of ≤20 ng/mL. For significant disease, NPV remained 62% and 61%, with a PPV of 85% and 100% at a cutoff level of 60 ng/mL and 120 ng/mL. Fifteen out of 98 patients had a value of 60 ng/mL or more, 2 cases with F1 fibrosis were falsely diagnosed as positive. All the 10 patients with a serum HA of 120 ng/mL or more had significant overall disease. In a previous study, in a cohort of 405 patients an AUROC of 0.73 was reported for significant fibrosis.[11] This is consistent with our results for the same category (AUROC=0.718). In another study, at a serum HA level of 60 mg/L, the PPV for the presence of fibrosis was found to be 93.9%, but NPV remained very low being only 50%.[17] The NPV remained low in our study too. This low NPV might not serve the purpose of identifying patients who can safely be deferred for an urgent treatment. In most of the previous studies, HA was evaluated for widespread fibrosis and cirrhosis.[18] Only a few studies have evaluated its predictive value in significant fibrosis and we could not find any study for overall disease category. Although, in our study, HA showed a better AUROC for activity grade, in the final analysis, we assessed HA for its value in overall disease category, which includes both fibrosis stage and activity grade. This histopathological category seems clinically more relevant, because both necroinflammatory activity grade and fibrosis stage are equally important in making treatment decisions. In addition, cross tabulation of our

patients for staging and grading, showed that the major determinant of overall significant disease category was the fibrosis stage, not the activity grade. Furthermore, we determined the discriminative value for significant versus minimal disease because this threshold is usually used for making treatment decisions when liver histology is known.[7]

Sutdy limitations. Most of the patients with minimal disease had F1 fibrosis, and those with significant disease had F2 fibrosis. Classification of fewer patients in extreme stages (F0 or F4) might have affected the discriminative value. The use of liver biopsy for the assessment of liver histology is not always possible because of its limitations and poor patient compliance. The risk of significant liver disease, on the other hand, makes treatment decisions difficult in an individual patient. Some recent noninvasive indexes consisting of HA in combination with other markers have shown promising results.[19,20] Similar noninvasive indexes need to be evaluated further in different populations.

In conclusion, serum HA is significantly associated with liver histology on biopsy when compared between minimal and significant disease groups. However, it may not be clinically useful in an individual for making treatment decisions, because of its low NPV for significant liver disease, and levels with clinically acceptable PPV found only in a small proportion of the patients.

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