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## Original Article

# Role of hyaluronic acid and laminin as serum markers for predicting significant fibrosis in patients with chronic hepatitis B

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### ABSTRACT

**Objectives:** The aim of this study was to evaluate the diagnostic performance of serum HA and LN as serum markers for predicting significant fibrosis in CHB patients.

**Methods:** Serum HA and LN levels of 87 patients with chronic hepatitis B and 19 blood donors were assayed by RIA. Liver fibrosis stages were determined according to the Metavir scoring-system. The diagnostic performances of all indexes were evaluated by the receiver operating characteristic (ROC) curves.

**Results:** Serum HA and LN concentrations increased significantly with the stage of hepatic fibrosis, which showed positive correlation with the stages of liver fibrosis (HA:  $r = 0.875$ ,  $p < 0.001$ ; LN:  $r = 0.610$ ,  $p < 0.001$ ). There were significant differences of serum HA and LN levels between F2-4 group in comparison with those in F0-F1 group ( $p < 0.001$ ) and controls ( $p < 0.001$ ), respectively. From ROC curves, 185.3 ng/mL as the optimal cut-off value of serum HA for diagnosis of significant fibrosis, giving its sensitivity, specificity, PPV, NPV, LR+, LR- and AC of 84.2%, 83.3%, 90.6%, 73.5%, 5.04, 0.19 and 83.9, respectively. While 132.7 ng/mL was the optimal cut-off value of serum LN, the sensitivity, specificity, PPV, NPV, LR+, LR- and AC were 71.9%, 80.0%, 87.2%, 60.0%, 3.59%, 0.35% and 74.7, respectively. Combinations of HA and LN by serial tests showed a perfect specificity and PPV of 100%, at the same time sensitivity declined to 63.2% and LR+ increased to 18.9, while parallel tests revealed a good sensitivity of 94.7%, NPV to 86.4%, and LR- declined to 0.08.

**Conclusions:** Serum HA and LN concentrations showed positive correlation with the stages of liver fibrosis. Detection of serum HA and LN in predicting significant fibrosis showed good diagnostic performance, which would be further optimized by combination of the two indices. HA and LN would be clinically useful serum markers for predicting significant fibrosis in patients with chronic hepatitis B, when liver biopsy is contraindicated.

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## Introduction

The prognosis and clinical treatment of chronic liver disease depends greatly on the progression of liver fibrosis, which is resulted from the loss of normal liver cell function due to disorganized over-accumulation of extra-cellular matrix (ECM) components in the liver.<sup>1-4</sup> In China, especially in our province, chronic hepatitis B is one of the main causes of chronic liver disease. Therefore it is clinically important to assess the progression of liver fibrosis for guiding clinical therapy.<sup>5</sup> For many years, liver biopsy has been considered the gold standard for staging and grading hepatic fibrosis and inflammation.<sup>1,6</sup> However, the procedure is associated with complications such as bleeding, infection, damage to liver tissue, etc,<sup>6,7</sup> and it is difficult to put into practice. Currently, with the improvements in treatment modalities for chronic liver disease, there is an increasing need for rapid, safe and reliable noninvasive diagnostic methods to stage liver fibrosis, and some of which have been widely validated in patients with chronic hepatitis.<sup>7-15</sup>

The ECM components in the liver include non-collagenous glycoproteins such as hyaluronic acid (HA), laminin (LN) and proteoglycans, etc. In the liver, HA is a glycosaminoglycan, which is mostly synthesized by hepatic stellate cells and degraded by sinusoidal endothelial cells.<sup>16</sup> LN is synthesized by hepatocytes and sinusoidal cells, which is one of the main glycoproteins of the basement membrane.<sup>17</sup> Reports showed that serum fibrosis indices, including HA and LN, could reflect the activity of liver fibrosis to some extent.<sup>6,18-26</sup>

In this study, we aimed to evaluate serum HA and LN levels as potential indicators of significant fibrosis in patients with chronic hepatitis B according to the Metavir scoring-system, as well as to assess the diagnosis performance of combination of the two indices.

## Methods

### Subjects

Eighty-seven patients with chronic hepatitis B, who were diagnosed by positive serologic tests for serum hepatitis B surface antigen for at least 6 months, were all from Affiliated Hospital of Nantong University, including 49 men and 38 women, median age (25<sup>th</sup> percentile; 75<sup>th</sup> percentile) 38.4 (34.1;49.9). These patients were included in this study with an indication for percutaneous liver biopsy, which was performed for assessment of the severity of liver fibrosis. Liver transplantation, gastrointestinal bleeding and other chronic liver diseases were excluded. Sera of 19 blood donors was used as a control group, including 10 men and 9 women, median age (25<sup>th</sup> percentile; 75<sup>th</sup> percentile): 30.1(28.1; 33.9). Healthy subjects had normal serum levels of regular biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferases (AST) and alkaline phosphatase (ALP), etc, and with no chronic liver diseases.

All patients provided written informed consent to participate in the study and Affiliated Hospital of Nantong University Ethical Committee approved the study.

### Histology

All liver biopsies were performed with a suction needle (18G, Angiomed Corporation – German). Ultrasound was routinely used to determine the percutaneous biopsy site. The size of liver biopsy specimen exceeded 1 cm. The liver tissue sections were fixed in 10% neutralized formaldehyde, embedded in paraffin and stained with hematoxylin-eosin, Mason-Trichrome, and Perls-iron. All biopsy specimens were analyzed by an experienced pathologist blinded to the clinical results of the patients. Liver fibrosis stages were evaluated semi-quantitatively according to the Metavir scoring-system.<sup>27</sup> Fibrosis was staged on a scale of 0 to 4: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis and few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis.

### Determination of serum specimens

All serum specimens from 87 patients with chronic hepatitis B were stored at -20°C. Levels of serum HA and LN were analyzed by RIA and determined from a standard curve. Kits of HA and LN were provided all by the Shanghai Navy Medical Institute. The procedures were performed according to the user's manual. Liver function indexes were measured by an automatic biochemistry analyzer, including ALT, AST, ALP, total bilirubin (TBIL),  $\gamma$ -glutamyltransferase (GGT) and albumin (ALB).

### Statistical analysis

The SPSS statistical package software (version 12.0) was used for statistical analysis.

Data are reported as the median with 25<sup>th</sup> and 75<sup>th</sup> percentiles of marker concentrations. Independent-samples t-test was used for analysis of the differences between two groups. Kruskal-Wallis one-way analysis of variance (ANOVA) was performed for comparing with the differences between patient subgroups (five stages of liver fibrosis) and healthy controls. Correlations were assessed by Spearman's correlation coefficient.

A two tailed p-value less than 0.05 was considered statistically significant. Sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratio and diagnostic accuracy were calculated according to the following formula (in which a = true-positive cases, b = false-positive cases, c = false-negative cases, and d = true-negative cases): sensitivity =  $a/(a + c)$ , specificity =  $d/(b + d)$ , diagnostic accuracy =  $(a + d)/(a + b + c + d)$ , positive predictive value =  $a/(a + b)$ , negative predictive value =  $d/(c + d)$ , positive likelihood ratio =  $\text{sensitivity}/(1 - \text{specificity})$ , negative likelihood ratio =  $(1 - \text{sensitivity})/\text{specificity}$ .

The ROC curves were constructed to study the absence and presence of significant fibrosis ( $F \geq 2$ ). The cut-off values were determined with ROC curve procedure.<sup>28</sup>

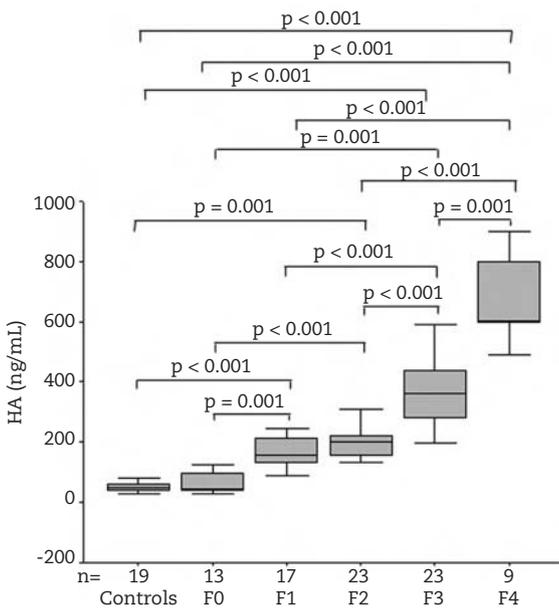
**Results**

According to the Metavir scoring-system, the fibrosis stages on liver biopsy was F0 in 13 patients (14.9%), F1 in 17 (19.5%), F2 in 25 (28.7%), F3 in 23 (26.4%), and F4 in 9 (10.3%) in this study. Thus, a total of 57 patients (65.5%) had significant fibrosis ( $\geq$  F2). The results through box plots showed that serum HA and LN concentrations increased significantly with the stage of hepatic fibrosis (Figs. 1 and 2), and the highest values of HA and LN were all found in cirrhotic patients. HA and LN levels showed positive correlation with the stages of liver fibrosis (HA:  $r = 0.875$ ,  $p < 0.001$ ; LN:  $r = 0.610$ ,  $p < 0.001$ ). The serum HA and LN concentrations did not differ significantly between the control group and the F0 group (HA:  $p = 0.604$ ; LN:  $p = 0.059$ ).

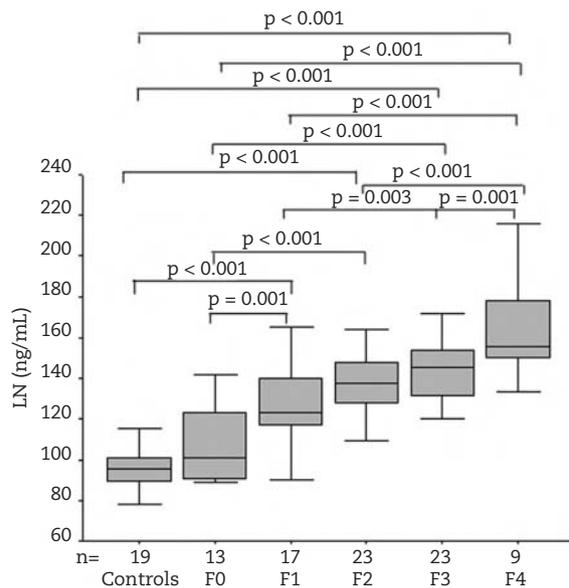
For significant fibrosis ( $F \geq 2$ ), Figs. 3 and 4 show the distribution of HA and LN concentrations in controls and CHB patients by fibrosis stage (F0-F1, F2-F4), respectively. Significant differences of serum HA and LN levels were found in F2-F4 group in comparison with those in F0-F1 group ( $p < 0.001$ ) and controls ( $p < 0.001$ ), respectively.

To predict significant fibrosis ( $F \geq 2$ ), the area under the curve (AUC) for serum HA and LN was 0.909 (95% CI: 0.847–0.971) and 0.815 (95% CI: 0.712–0.917), respectively (Fig. 5). The difference was not statistically significant ( $p = 0.743$ ). The results showed that 185.3 ng/mL was the optimal cut-off value of serum HA for diagnosis of significant fibrosis, giving its sensitivity, specificity, PPV, NPV, LR+, LR- and AC of 84.2%, 83.3%, 90.6%, 73.5%, 5.04, 0.19 and 83.9, respectively. When choosing 132.7 ng/mL as the optimal cut-off value of serum LN, the sensitivity, specificity, PPV, NPV, LR+, LR- and AC were 71.9%, 80.0%, 87.2%, 60.0%, 3.59%, 0.35% and 74.7, respectively. Details were shown in Table 1, in which the performance of combinations of HA and LN by serial and parallel tests has also been shown respectively. Serial tests showed a perfect specificity and PPV of 100%, but at the same time sensitivity declined to 63.2% and LR+ increased to 18.9. Parallel tests revealed a good sensitivity of 94.7%, NPV to 86.4%, while LR- declined to 0.08. In Table 2, some serum biochemical indexes were chosen as the predictors of significant fibrosis, none of which had shown high diagnostic performance.

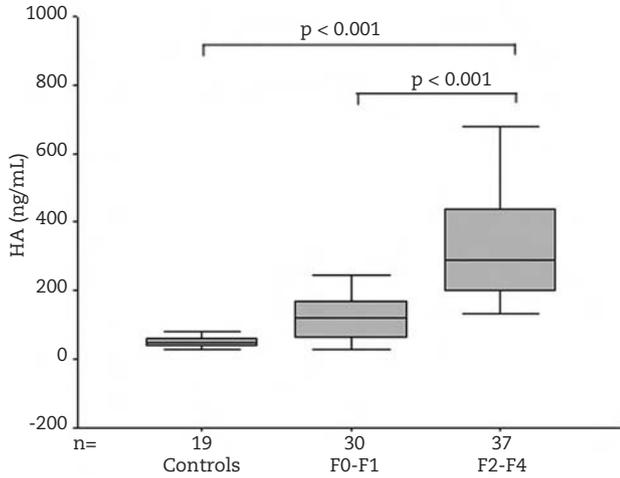
Fig. 6 was an In/In plot of serum HA levels vs. serum LN concentration for all 87 CHB patients. In Fig. 6, the capability of two variables to discriminate completely between F2-F4 group and F0-F1 group was readily appreciable.



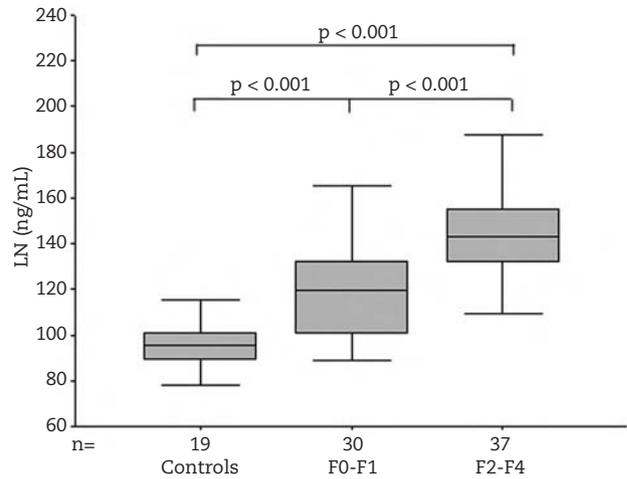
**Fig. 1 - Box plots illustrating the distribution of HA values in controls and CHB patients against METAVIR fibrosis score (F0-F4). The line through the middle of each box represents median value, and the lower and upper borders of the box encompass the interquartile range. The error bars are the 5<sup>th</sup> and 95<sup>th</sup> percentiles.**



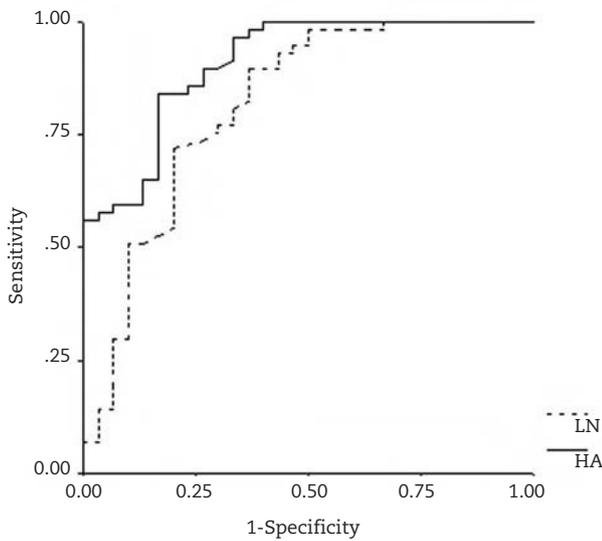
**Fig. 2 - Box plots illustrating the distribution of LN values in controls and CHB patients against METAVIR fibrosis score (F0-F4). The line through the middle of each box represents median value, and the lower and upper borders of the box encompass the interquartile range. The error bars are the 5<sup>th</sup> and 95<sup>th</sup> percentiles.**



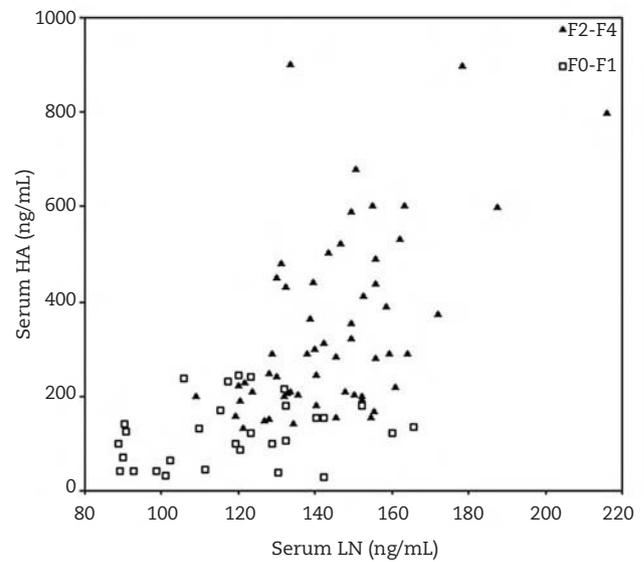
**Fig. 3 - Distribution of HA values in blood donors (controls) and CHB patients by fibrosis stage (F0-F1, F2-F4). The line through the middle of each box represents median value, and the lower and upper borders of the box encompass the interquartile range. The error bars are the 5<sup>th</sup> and 95<sup>th</sup> percentiles.**



**Fig. 4 - Distribution of LN values in blood donors (controls) and CHB patients by fibrosis stage (F0-F1, F2-F4). The line through the middle of each box represents median value, and the lower and upper borders of the box encompass the interquartile range. The error bars are the 5<sup>th</sup> and 95<sup>th</sup> percentiles.**



**Fig. 5 - Receiver-operating characteristic (ROC) curves of serum HA and LN for diagnosis of significant fibrosis (F ≥ 2).**



**Fig. 6 - Serum HA vs serum LN concentrations in all 87 CHB patients with F0-F1 group (n = 30) or with F2-F4 group (n = 57).**

**Table 1 - Diagnostic performance of the best cut-off point for serum levels of HA and LN in the prediction of significant fibrosis ( $F \geq 2$ ) for CHB patients**

Variables	AUC	CI (95%)	Cut-off (ng/mL)	Se (%)	Sp (%)	PPV (%)	NPV (%)	LR +	LR -	AC (%)
HA	0.909	0.847-0.971	185.3	84.2	83.3	90.6	73.5	5.04	0.19	83.9
LN	0.815	0.712-0.917	132.7	71.9	80	87.2	60	35.9	0.35	74.7
HA + LN (Serial tests)				63.2	100	100	58.9	18.9	0.38	75.9
(Parallel tests)				94.7	63.3	83.1	86.4	2.6	0.08	83.9

AUC, area under the curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio for positive test; LR-, likelihood ratio for negative test; AC, accuracy.

**Table 2 - Diagnostic performance of serum biochemical indexes in the prediction of significant fibrosis ( $F \geq 2$ ) for CHB patients**

Variables	AUC	Cut-off (ng/mL)	Se (%)	Sp (%)	PPV (%)	NPV (%)	LR +	LR -	AC (%)
ALT	0.698	62.5 (U/L)	69.2	58.6	70.7	52.6	2.4	0.41	65.7
AST	0.671	50.2 (U/L)	75.1	53.5	68.6	56.2	2.5	0.40	68.6
GGT	0.590	47.2 (U/L)	55.3	68.8	70.5	51.5	1.8	0.62	59.8
ALP	0.573	102.5 (U/L)	50.1	70.2	49.8	70.0	1.6	0.59	64.3
ALB	0.801	36.1 (g/L)	77.2	69.8	86.5	73.2	3.3	0.36	74.5
TBIL	0.711	25.2 ( $\mu$ mol/L)	58.9	80.2	83.9	49.3	3.0	0.37	64.1

AUC, area under the curve; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio for positive test; LR-, likelihood ratio for negative test; AC, accuracy.

## Discussion

In China, especially in our province, chronic hepatitis B is one of the main causes of chronic liver disease. Therefore, it is clinically important to assess the progression of liver fibrosis for guiding clinical therapy.<sup>5</sup> Liver biopsy fails to satisfy the more and more pronounced need for a rapid, safe and repeatable tool to monitor the fibrogenic progression of chronic liver disease. The ideal surrogate blood markers should enable repetitive measurement and be provided with other features, such as liver specificity, sensitivity for fibrogenesis/fibrolysis, known half-life, known excretion route, synthesis by an identified cell source, etc.<sup>29</sup> In this study, we aimed to assess the usefulness of serum HA and LN as biomarkers for predicting significant fibrosis in CHB patients with ROC curves. We found that as liver fibrosis progresses, there is a significantly increase of serum HA and LN concentrations correspondingly, and the highest values of HA and LN were all found in cirrhotic group ( $F = 4$ ). In addition, HA and LN levels showed positive correlation with the stages of liver fibrosis in CHB patients (HA:  $r = 0.875$ ,  $p < 0.001$ ; LN:  $r = 0.610$ ,  $p < 0.001$ ). The results suggested that increased serum HA and LN levels, which are components of ECM, might indicate a consequence of chronic liver injury, leading to architectural changes in the liver parenchyma that causes liver fibrosis eventually.

As one component of ECM, HA has been described as a single parameter or as a major member of several fibrosis indexes for the noninvasive assessment of fibrosis in various

chronic liver diseases in the past few years.<sup>6,10,11,19,24,30</sup> In our study, HA levels at its best cut-off value of 185.3 ng/mL for predicting significant fibrosis showed a high diagnostic performance, especially AUC-ROC of 0.909. For a certain marker, a value more than 0.9 for AUC-ROC means it is possible to differentiate between the two compared groups through this marker, while less than 0.7 for it means that it is not possible to differentiate between them.<sup>31</sup> It suggested that HA as a biomarker could predict significant fibrosis at its optimal cut-off value.

In recent years, detection of serum LN was usually as a member of combined analysis of several fibrosis indexes rather than assay of only LN levels in liver fibrosis.<sup>1,10,11,32-34</sup> Our results showed that detection of serum LN concentrations was moderately accurate at the diagnosis of significant fibrosis (AUC-ROC of 0.815), but NPV was only 60%, which could be unacceptable for excluding significant fibrosis. Thought it may be explained due to the low number of patients, the diagnosis performance of LN was not better than that of HA, according to this study.

We also assessed the performance of combined HA and LN for predicting significant fibrosis. The results of serial tests showed a perfect specificity and PPV of 100%, LR+ up to 18.9, which could be acceptable for the definite diagnosis of significant fibrosis. At the same time, the results of parallel tests showed that sensitivity was raised to 94.7%, NPV to 86.4%, and LR- declined to 0.08, thus we could make a diagnosis for excluding significant fibrosis.

In conclusion, increased serum HA and LN concentrations showed positive correlation with the stages of liver fibrosis. Detection of serum HA and LN in prediction of significant fibrosis showed good diagnostic performance, which would be further optimized by combination of the two indices. HA and LN would be clinically useful serum markers for predicting significant fibrosis in patients with chronic hepatitis B, when liver biopsy is contraindicated.

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## Conflict of interest

All authors declare to have no conflict of interest.

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