PILOT STUDY

The relationship of Serum Histone H3.3 and H4 with chronic Hepatitis B

Ali Tuzun Ince[1], Elmas Biberci Keskin[2], Bilge Sumbul Gultepe[3], Koray Kochan[4], Ibrahim Hakkı Koker[5], Hakan Senturk[6]

Abstract

Objective: To determine the role of serum histone H3.3 and H4 in patients with chronic hepatitis B to explore any relationship between the two.

Methods: The prospective controlled clinical pilot study was conducted in the Gastroenterology Clinic of Bezmialem Vakif University, Istanbul, Turkey, from January to October 2017, and comprised biopsy-proven patients with chronic hepatitis B and healthy controls. Demographics, hepatitis B virus deoxyribonucleic acid quantity, hepatitis B e-antigen, aspartate aminotransferase, alanine transaminase, international normalized ratio, total/direct bilirubin, albumin and thrombocyte counts as well as histological activity index and fibrosis scores were noted. Data was analysed using SPSS 22.

Results: Of the 140 subjects, 70(50%) each were cases and controls. The overall mean age of the sample was 43.38±15.07 years (range: 18-70 years). There was positive correlation of histone H3.3 with hepatitis B virus deoxyribonucleic acid, aspartate aminotransferase, alanine transaminase and international normalized ratio levels. Histone H4 levels only correlated with hepatitis B virus deoxyribonucleic acid and international normalized ratio. Hepatitis B e-antigen positivity was present in 14(20%) of the cases.

Conclusion: Histone H3.3 levels appeared to be associated with pathophysiological changes in chronic hepatitis B patients, suggesting that future treatments should target H3.3.

Keywords: Histone H3.3, Histone H4, Extracellular histone, Chronic Hepatitis B, HBV.
(JPMA 70: 1596; 2020) **DOI: https://doi.org/10.5455/JPMA.19365**

Introduction

More than 250.000.000 people have been living with chronic hepatitis B (CHB) infection in the world and 887,000 people lost their lives in 2015 because of complications of this disease, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Approximately 5-10% of adults with acute hepatitis B will become chronic.[1]

A nucleosome consists of deoxyribonucleic acid (DNA) wrapped around eight histone proteins (double copies every H2A, H2B, H3, and H4 proteins).[2] Histones are the positively-charged basic building blocks of the chromatins that hold the DNA together. The main sources of histones in the serum are immune and parenchymal cells. They have various physiological and pathogenic properties. Their main physiological roles are remodelling of chromatins and gene transcription.[3,4] When cells are activated or destroyed during apoptosis, they get released into the extracellular environment, and produce toxic and inflammatory effects. They also work as endogenous damage-associated molecular patterns (DAMPs). Histones play roles in pathogenic properties, such as apoptosis facilitation of microvascular dysfunction in sepsis,[5] thrombin generation, coagulation and thrombogenesis in atherosclerosis,[5] CHB,[6,7] trauma, tissue injury, such as liver, kidney or lung,[8,9,10,11] autoimmune and inflammatory diseases as well as cancer.[12] Increased levels of histones have been demonstrated in all of these pathological states.

Notably, histones show antimicrobial and antiviral activity, mostly by accumulating in cytoplasm and on the plasma membrane.[13,14] Recently, a study demonstrated the neutralisation of influenza viruses by histones H3 and H4: specifically, the antiviral effect of H4 is explained by direct interaction with the virus.[15] Some studies have suggested that extracellular histones may work on markers for the diagnosis, prognosis and management of various diseases.[16] Histone neutralising antibody treatment or elimination of Toll-like receptor (TLR) 2, TLR4 and TLR9 in mice protects against histone-mediated liver injury.[18]

Covalently closed circular DNA (cccDNA) is a repeated form of the hepatitis B virus (HBV) DNA. It persists within the nuclei of infected liver cells, produces viral ribonucleic acid (RNA) transcripts, and is difficult to eradicate. HBV cccDNA accumulates in the cells as a mini-chromosome, which contains both histone (large amounts of H3 and H2B and small amounts of H4, H2A, and H1) and non-histone proteins.[5] It has been shown that the incidence of cccDNA transcription-associated H3 and H4 histones is about 10-fold lower in patients who are hepatitis B e-antigen (HBeAg)-negative than in positive ones.

Histone H3 is 15-16 kDa protein consisting of ~135 amino acid residues. H3-H4 heterotetramer forms a core

[1,2,4,5,6]Department of Internal Medicine, Gastroenterology Clinic of Bezmialem Vakif University, Istanbul, Turkey; [3]Department of Microbiology, Bezmialem Vakif University, Istanbul, Turkey.
Correspondence: Ali Tuzun Ince. e-mail: dralince@gmail.com

component of the nucleosome. The human histone H3.3, an H3 variant found in all eukaryotes, is replication- and cell cycle phase-independent and is the most common H3 variant type in non-dividing cells.[17] There have been 216 different members reported from diverse species, and 6 different variants of human histone H3; Histone H3-like centromeric protein A, H3.1, H3.1t, H3.2, H3.3 and H3.3C. Studies have shown that covalent modifications of H3.3 are associated with gene activation.[18,19]

The current study was planned to see whether there is a correlation of serum histone H3.3 and H4 with viral, biochemical tests and pathology results in patients with CHB.

Patients and Methods

The prospective controlled clinical pilot study was conducted in the Gastroenterology Clinic of Bezmialem Vakif University, Istanbul, Turkey, from January to October 2017. After approval from the institutional ethics committee, liver biopsy-proven CHB patients aged 18–70 years were enrolled as the study group and an equal group of age-matched healthy subjects were taken as the control group. Patients were selected from among the outpatient clinic. Those excluded were patients with generalised impairment, sepsis, severe heart, kidney, or liver failure, any other acute or chronic liver disorder, including toxic liver disease, which is another form of viral hepatitis C or A, autoimmune hepatitis, hemochromatosis, Wilson's disease, and any co-infection, like hepatitis D virus (HDV), hepatitis C virus (HCV), or human immunodeficiency virus (HIV). Written informed consent was obtained from all the subjects.

The diagnosis of CHB infection was made if the HBV infection had persisted for ≥6 months, the HBV DNA level was elevated (≥2.000IU/mL in HBeAg-negative patients and ≥20.000IU/ml in HBeAg-positive patients, alanine aminotransferase (ALT) levels was increased, and liver biopsy was confirmatory.

Blood samples 10mL were obtained and placed in tubes containing sodium citrate and centrifuged for 20min at 3,000g. Serum and plasma samples were stored at -80^{O}C prior to analysis. Baseline and demographic characteristics, like age and gender, levels of aspartate aminotransferase (AST), ALT, albumin, total and direct bilirubin, international normalised ratio (INR), and thrombocyte count (/mm^3) were recorded prospectively, as were the HBV DNA level (IU/mL) and HBeAg status. Histological activity index (HAI) and fibrosis scores were obtained using the Knoddle system.[20] The HAI was classified as mild (score of 0–6), moderate (7–12), or severe (13–18). Similarly, fibrosis was classified as mild (score of 0–2), moderate (3–4) or severe (5–6). Histone H3.3 and H4 levels were measured using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, Inc. San Diego, CA, USA) in 96-well plates pre-coated with anti-human histone H3.3 and histone H4 acetylated on lysine 16 (HIST1H4) antibodies, according to the manufacturer's instructions. Biotin-conjugated anti-H3.3 and anti-HIST1H4A antibodies were used for detection. Standards, test samples and biotin-conjugated detection antibodies were later added to the wells, followed by washing. Horseradish peroxidase (HRP)-streptavidin was then added and the unbound conjugates were removed by washing. To visualise the enzymatic reaction, 3,3',5,5'-tetramethylbenzidine (TMB) was used. In the presence of HRP, TMB generated a blue product which was converted to a yellow compound after the addition of acidic stop solution. The intensity of yellow was proportional to the amounts of histone H3.3 and HIST1H4A. Absorbance was read at 450nm using a microplate reader and histone concentrations were calculated in ng/mL using standard curves.

Data was analysed using SPSS 22. Power analysis of the study was done earlier using G*Power software.[21] As no similar prior study has been performed, the power analysis was based on a pilot study comprising 15 subjects. The effect size 'd' was 0.484 and the standard deviation (SD) of the H4ng parameter 1.6; thus, a minimum of 68 subjects in each group were required for a power 0.80 and an alpha value of 0.05. Data normality was evaluated using the Shapiro-Wilks test. Student's t-test and Mann-Whitney U test were employed to compare inter-group data that was normally and non-normally distributed, respectively. Qualitative data were compared using chi-squared test. Spearman's rho correlations were derived to explore the relationships between parameters that were not normally distributed. $P<0.05$ indicated significance.

Results

Of the 140 subjects, 70(50%) each were cases and controls. The overall mean age of the sample was 43.38±15.07 years, and there were 62(44.3%) females and 78(55.7%) males. The mean age did not differ between the groups ($p>0.05$). Males were more likely to be in the CHB-infected group than the control group ($p=0.041$). HAI, fibrosis scores and HBeAg-positivity of the cases were also noted (Table 1).

There was no significant inter-group differences in gender or the levels of histones H3.3 or H4 ($p>0.05$). Although histone H3.3 levels were much higher in the patients than in the controls ($p\leq0.001$), the same was not the case for H4 levels ($p>0.05$). Histone H3.3 level was higher in HBeAg-positive than HBeAg-negative patients ($p\leq0.001$). No significant relationship was found between histone H4

Table-1: Patient demographics, hepatitis B e-antigen (HBeAg) status, and the histological activity index and fibrosis scores.

		Patients	Controls	p-value
Mean Age (years)		42.50 ± 11.64	44.26 ± 17.91	[1]0.493
Gender (n, %)	Female	25 (35.7%)	37 (52.9%)	[2]0.041*
	Male	45 (64.3%)	33 (47.1%)	
HBeAg status (n, %)	Positive	14 (20%)		
	Negative	56 (80%)		
Histologic activity index (n, %)	Mild	25 (35.7%)		
	Moderate	43 (61.4%)		
	Severe	2 (2.9%)		
Fibrosis (n, %)	Mild	62 (88.6%)		
	Moderate	4 (5.7%)		
	Severe	4 (5.7%)		

[1]Student's t-test, [2]chi-squared test, *: p<0.05

level and HBeAg-positive status (p>0.05) (Table 2).

Positive correlations were evident between histone H3.3 level and the levels of HBV DNA (p≤0.001), AST (p≤0.001), ALT (p≤0.001) and INR (p=0.020). However, no relationship was apparent between H3.3 level and HAI score, fibrosis score, age, albumin, total bilirubin, direct bilirubin, or platelet levels (p>0.05).

Positive relationships were evident between histone H4 level and the level of HBV DNA (p=0.014) and INR (p=0.021), but not between histone H4 level and age, HAI score, fibrosis score, AST, ALT, albumin, total bilirubin, direct bilirubin, or platelet levels (p>0.05) (Table 3).

While histone H3.3 level rose as CHB progressed, H4 level did not, and there was no significant correlation of histone H3.3 or H4 levels with HAI or fibrosis scores, AST, ALT, albumin, total bilirubin, direct bilirubin and platelet levels (p>0.05). However, histone H3.3 and H4 levels correlated with INR (p<0.05).

Table-2: Histone H3.3 and H4 distributions by gender, study group, and hepatitis B e-antigen (HBeAg) status.

		Histone H3.3	Histone H4
		Mean ± SD (median) (min to max)	
Gender	Female	6,841,74 ± 13,083.03 (1937.9) (0-59028)	0.44 ± 1.46 (0) (0-7.1)
	Male	36,260.07 ± 149,196.2 (2,843.1) (0-933394.2)	0.35 ± 1.12 (0) (0-5.9)
	p-value	0.305	0.620
Study group	HBV	25,753.53 ± 120,231.36 (2,648.3) (0-933394.2)	0.38 ± 1.24 (0) (0-7.1)
	Control	83.05 ± 14.69 (83.6) (45.628-111.8)	0.07 ± 0.26 (0) (0-1.5)
	p-value	≤ 0.001*	0.697
HBeAg status	Positive	10,1471.6 ± 261 530.81 (6,755.2) (0-933394.2)	0.71 ± 1.64 (0) (0-5.9)
	Negative	6824 ± 12 003.57 (2 369.8) (0-59028)	0.3 ± 1.13 (0) (0-7.1)
	p-value	0.046*	0.065

Mann Whitney U test; SD: standard deviation; *: p<0.05, SD: Standard deviation; HBV: Hepatitis B virus

Table-3: Correlations between histone H3.3 and H4 levels and other parameters in the chronic hepatitis B-infected group.

		H3.3	H4
Age	R_s	-0.008	-0.085
	p-value	0.948	0.484
HBV DNA (IU/mL)	R_s	0.463	0.293
	p-value	≤0.001*	0.014*
Histological activity index	R_s	0.233	0.088
	p-value	0.052	0.468
Fibrosis score	R_s	0.137	0.175
	p-value	0.257	0.148
AST level	R_s	0.604	0.055
	p-value	≤0.001*	0.649
ALT level	R_s	0.536	0.050
	p-value	≤0.001*	0.683
Albumin level	R_s	-0.141	0.002
	p-value	0.245	0.988
INR	R_s	0.278	0.276
	p-value	0.020*	0.021*
Total bilirubin level	R_s	-0.079	0.000
	p-value	0.517	0.998
Direct bilirubin level	R_s	0.054	0.063
	p-value	0.656	0.607
Thrombocyte count	R_s	-0.091	0.081
	p-value	0.452	0.507

Spearman's rho correlation analyses; *: p<0.05; HBV: Hepatitis B virus; DNA: Deoxyribonucleic acid; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR, International normalised ratio; R_s value The Spearman's Rank Correlation Coefficient.

Discussion

In the current study, histone H3.3 and H4 levels correlated with the HBV DNA level and INR. Histone H3.3 level also correlated with AST and ALT levels, and with HBeAg-positive status. However, we found no correlation between histone H3.3 or H4 levels and age, HAI/fibrosis scores, or albumin, total bilirubin, direct bilirubin, or platelet levels. No relationships were apparent between the HAI or fibrosis scores and the levels of H3.3 or H4.

Extracellular histones, the levels of which are negatively prognostic for several diseases, are promising targets for treating CHB. Earlier studies explored the relationship between histone levels and CHB infection, but most focussed on histone H4 rather than histone H3.3, although the histone H4 level may serve as a surrogate of the total histone concentration.[22] Serum histone levels increase in animals with liver injuries.[9] Significant increases were demonstrated in an in vivo model of hepatic ischemia/reperfusion injury, wherein histone neutralisation significantly protected against injury.[23] Li et al. evaluated histone H4

status in patients with acute liver failure (ALF), CHB and LC, as well as in healthy controls.[24] The H4 level increased significantly in ALF patients, but was only slightly elevated in CHB and LC patients compared to the healthy controls. No significant correlations between the levels of histones and cytokines, such as interleukin [IL]-6, IL-8, and IL-10 were evident in CHB-infected or LC patients compared to healthy controls. It was concluded that extracellular histones did not play a central role in chronic liver disease. Wein et al.[25] reported similar results. Kawai et al.[26] showed that histone H3 was deposited along liver capillaries after injection into mice, compromising liver function.

The current study found that histone H3.3 and H4 levels correlated with HBV DNA level and the INR. Further, cccDNA is thought to be responsible for CHB infection onset and persistence after antiviral treatment. As HBV cccDNA forms mini-chromosomes in the nuclei of infected cells, and as the DNA binds both histone and non-histone proteins, a positive relationship of HBV DNA level with histone H3.3 and 4 levels would be expected. Histone H3.3 levels increased markedly in CHB-infected HBeAg-positive patients compared to healthy controls. Similar to Li et al., we found that the histone H4 level in CHB-infected patients was not higher than that in healthy controls,[24] possibly because histone H3.3 is more abundant than histone H4 in HBV mini-chromosomes. The histone H3.3 level correlated with AST and ALT levels. As CHB infection progressed, increased production and release of histone H3.3 into the extracellular space further damaged the liver, increasing AST and ALT levels. However, we found no correlations of histone H3.3 or H4 levels with age, HAI/fibrosis scores, or albumin, total bilirubin, direct bilirubin, or platelet levels. We expected that the HAI score and histone H3.3 level would be positively correlated in CHB patients. It is possible that the severity of inflammation in our CHB patients was lower than that of the acute liver failure (ALF) patients studied by Li et al.[24] If ALF is associated with severe inflammation, a significant increase in serum histone levels would be expected. As only a few of our patients had severe fibrosis, we found no correlation between this score and histone levels, unlike Li et al.[24] Although we found a correlation between histone levels and INR, no correlations of histone H3.3 or H4 levels with the albumin level or thrombocyte count were seen; the latter parameters, as well as INR, are affected by LC because the number of viable liver cells releasing extracellular histones H3.3 and H4 is reduced, and in some LC patients these cells are entirely absent. Thus, we principally included patients with mild to moderate HAI and fibrosis scores. Both Voltz et al. and Laras et al. found 10-fold greater amounts of HBV cccDNA in HBeAg-positive versus HBeAg-negative patients.[6,7] Similarly, we found a correlation between HBeAg-positive status and histone H3.3 level. Both the HBV DNA level and the INR reflected the histone H3.3 and H4 levels. We found positive correlations between histone H3.3 level and AST and ALT levels, INR and HBeAg-positive status, reflecting HBV-induced damage to liver cells. We found no relationship between HAI or fibrosis scores and the histone H3.3 or H4 level.

As the histone H3.3 level rises with progression of CHB infection, but not with progression of LC, the histone H3.3 level can be used to distinguish active disease from harmless viral carriage. Approximately one-third of CHB-infected patients exhibit normal liver enzyme levels, but with elevated HBV levels, and pathological examination of liver samples may reveal a need for treatment. In future, biopsy may not be required and novel treatments could target histone H3.3 of HBV cccDNA.

The limitations of the current study included a relatively small sample size, and the fact that the HAI scores were generally mild to moderate. The serum histone H3.3 level was elevated in CHB-infected patients. The potential roles of the five other histones should be investigated in future studies.

Conclusion

Targeting of histone H3.3 on cccDNA of HBV minichromosomes may be useful to treat CHB infection. The histone H3.3 level can be used to differentiate active disease from the harmless carriage stage without any need for biopsy.

Disclaimer: The study was presented as an Oral Presentation at the 35th National Gastroenterology Week, Antalya, Turkey, by us in November 2018.'

Conflict of interest: None.

Source of Funding: Bezmialem Vakif University Scientific Council, Istanbul, Turkey.

References

1. World Health Organization. Global hepatitis report 2017.
2. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. Nature. 1997; 389: 251-60.
3. Bhaumik SR, Smith E, Shilatifard A. Covalent modifications of histones during development and disease pathogenesis. Nat Struct Mol Biol. 2007; 14:1008-16.
4. Zentner GE, Henikoff S. Regulation of nucleosome dynamics by histone modifications. Nat Struct Mol Biol. 2013; 20:259-66.
5. Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, et al. Structural organization of the hepatitis B virus minichromosome. J Mol Biol. 2001; 307:183-96.
6. Volz T, Lutgehetmann M, Wachtler P, Jacob A, Quaas A, Murray JM, et al. Impaired intrahepatic hepatitis B virus productivity contributes to low viremia in most HBeAg-negative patients. Gastroenterology. 2007; 133: 843-52.

4. Laras A, Koskinas J, Dimou E, Kostamena A, Hadziyannis SJ. Intrahepatic levels and replicative activity of covalently closed circular hepatitis B virus DNA in chronically infected patients. Hepatology. 2006; 44: 694-702.
8. Xu J, Zhang X, Monestier M, Esmon NL, Esmon CT. Extracellular histones are mediators of death through TLR2 and TLR4 in mouse fatal liver injury. J Immunol. 2011; 187:2626-31.
9. Wen Z, Liu Y, Li F, Ren F, Chen D, Li X, et al. Circulating histones exacerbate inflammation in mice with acute liver failure. J Cell Biochem. 2013; 114: 2384-91.
10. Allam R, Scherbaum CR, Darisipudi MN, Mulay SR, Hägele H, Lichtnekert J, et al. Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. J Am Soc Nephrol. 2012; 23:1375-88.
11. Bosmann M, Grailer JJ, Ruemmler R, Russkamp NF, Zetoune FS, Sarma JV, et al. Extracellular histones are essential effectors of C5aR- and C5L2-mediated tissue damage and inflammation in acute lung injury. Faseb J. 2013; 27: 5010-21.
12. Allam R, Kumar SV, Darisipudi MN, Anders HJ. Extracellular histones in tissue injury and inflammation. J Mol Med (Berl). 2014; 92: 465-72.
13. Zlatanova JS, Srebreva LN, Banchev TB, Tasheva BT, Tsanev RG. Cytoplasmic pool of histone H1 in mammalian cells. J Cell Sci. 1990; 96: 461-8.
14. Watson K, Edwards RJ, Shaunak S, Parmelee DC, Sarraf C, Gooderham NJ, et al. Extra-nuclear location of histones in activated human peripheral blood lymphocytes and cultured T-cells. Biochem. Pharmacol. 1995; 50:299-309.
15. Hoeksema M, Tripathi S, White M, Qi L, Taubenberger J, van Eijk M, et al. Arginine-rich histones have strong antiviral activity for influenza A viruses. Innate Immun. 2015; 21:736-45.
16. Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. Cell Death Dis. 2014; 5: e1370.
17. Gabrielli F, Aden DP, Carrel SC, von Bahr C, Rane A, Angeletti CA, et al. Histone complements of human tissues, carcinomas, and carcinoma-derived cell lines. Mol Cell Biochem. 1984; 65:57-66.
18. McKittrick E, Gafken PR, Ahmad K, Henikoff S. Histone H3.3 is enriched in covalent modifications associated with active chromatin. Proc Natl Acad Sci U S A. 2004; 101:1525-30.
19. Hake SB, Garcia BA, Duncan EM, Kauer M, Dellaire G, Shabanowitz J, et al. Expression patterns and post-translational modifications associated with mammalian histone H3 variants. J Biol Chem. 2006; 281: 559-68.
20. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, et al. Formulation and Application of a Numerical Scoring System for Assessing Histological Activity in Asymptomatic Chronic Active Hepatitis. Hepatology 1981; 1:431-5.
21. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007; 39: 175-91.
22. Ekaney M, Otto G, Sossdorf M, Sponholz C, Boehringer M, Loesche W, et al. Impact of plasma histones in human sepsis and their contribution to cellular injury and inflammation. Crit Care. 2014; 18:543.
23. Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. Hepatology. 2011; 54: 999-1008.
24. Li X, Gou C, Yao L, Lei Z, Gu T, Ren F, et al. Patients with HBV-related acute-on-chronic liver failure have increased concentrations of extracellular histones aggravating cellular damage and systemic inflammation. J Viral Hepat. 2017; 24: 59- 67.
25. Wen Z, Lei Z, Yao L, Jiang P, Gu T, Ren F, et al. Circulating histones are major mediators of systemic inflammation and cellular injury in patients with acute liver failure. Cell Death Dis. 2016; 7: e2391.
26. Kawai C, Kotani H, Miyao M, Ishida T, Jemail L, Abiru H, et al. Circulating Extracellular Histones Are Clinically Relevant Mediators of Multiple Organ Injury. Am J Pathol. 2016;186:829-43.