Original Article

Hepatitis B Pre-genomic RNA as Biomarkers for the Evaluation of Liver Fibrosis and Severity of the Disease in Patients with Chronic Hepatitis B Infection

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Abstract

Background: The key reason for the persistence of the hepatitis B virus (HBV) is intrahepatic covalently closed circular DNA (cccDNA). Therefore, a noninvasive serum biomarker that can indicate intrahepatic cccDNA is necessary for assessing the virological, biochemical, and therapeutic responses to HBV. **Objectives:** To measure the level of HBV pre-genomic RNA (pgRNA), associate the measured levels of the pgRNA with that of the fibro scan-based liver fibrosis stage, and correlate with the HBV DNA load and liver enzymes. Monitor Hepatitis B,E Antigen status to compare with the level of HBV-pgRNA. **Materials and Methods:** Upto 89 patients with persistent HBV infection underwent fibro scan-based liver fibrosis staging, F0, F1, F2, F3, and F4. Their liver fibrosis was monitored by a fibroscan device along with relevant data such as fibroscan staging qualitative HBsAg, and liver enzymes were derived from medical records of patients who were recruited according to the specialist decision. Some of these data were provided by direct interviewing with patients including age, sex, duration of taking antiviral therapy, and duration of the disease. In order to characterize HBV RNA in plasma, we measured its concentration, DNA (viral load), and the quantity of HBsAg, HBe Ag status. For the detection of HBV RNA and DNA in plasma, total nucleic acid was isolated from plasma and separated into two tubes: the first one was treated with DNAse 1 to degrade DNA and the remaining RNA was reverse transcribed into cDNA. HBV DNA and HBV RNA were quantified together using a real-time qPCR technique targeting a conserved segment of the core region after being treated with RNAse enzyme. For HBeAg, qualitative ELISA assay technique was used to determine the status of HBeAg. **Results:** A total of 89 patients with chronic hepatitis B infection (52 males and 37 females) with a range age of 18–80 years who had undergone fibroscan staging 42 (47.2%F0), 20 (22.5%F1), 11 (12.4%F2), 11 (12.4%F3), and 5 (5.6%F4). Regarding HBeAg status, 94.4% were negative and 5.6% were positive. HBeAg was found to be significantly associated with the staging of fibrosis ($P \leq 0.01$); RNA level was intermediate directly correlated and highly significantly associated DNA levels ($R = 0.31$, $P \leq 0.01$), level of HBeAg was significantly associated with severity of fibrosis. HBeAg was found to be significantly associated with the staging of fibrosis ($P \leq 0.01$). **Conclusions:** pgRNA is a promising biomarker to predict liver fibrosis as it is a surrogate biomarker for cccDNA.

Keywords: cccDNA, CHBV, fibro scan, pre-genomic RNA

Introduction

Hepatitis B virus (HBV) infection affects roughly 2.57 billion people worldwide, and it causes about 750,000 deaths annually. More than 250 million of them are chronically infected, which increases the risk of developing HBV-related liver diseases such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[1] The family hepadnaviridae includes the enveloped DNA virus known as HBV. The 3.2 kb relaxed circular (rc) DNA genome of the virion is partially double-stranded (ds). The viral rcDNA enters the nucleus after infecting a hepatocyte via the hepatocyte-specific receptor NTCP to create an episomal covalently closed circular DNA (cccDNA), which resembles a minichromosome. The 3.5-kb precore (preC)

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and pre-genomic (pg) RNA, 2.4/2.1-kb surface mRNAs, and 0.7-kb X mRNA are among the five overlapping 3′ end mRNAs that cccDNA produces using the host RNA polymerase II. HBV replicates its DNA genome in the cytoplasmic nucleocapsid by protein-primed reverse transcription of pre-genomic RNA (pgRNA), which is catalyzed by viral polymerase. In order to produce a progeny virion, the freshly manufactured rcDNA-containing nucleocapsid is enveloped by viral surface proteins and released through the cell's multivesicular body (MVB) secretory route.

In addition to the DNA-containing virions, an excess of nucleocapsid-free sub-viral particles that self-assembled in the endoplasmic reticulum (ER) lumen is released through the endogenous secretory pathway, which accumulates extracellular as HBsAg.[2,3] pgRNA is transcribed utilizing cccDNA as a template in the viral nucleocapsid and is a byproduct of HBV replication[4] pgRNA not only the direct transcription product of cccDNA but also can encode HBV polymerase which can convert pgRNA into rcDNA. The rcDNA can be then repaired to form HBV cccDNA.[5] For all HBV messenger RNAs (mRNAs), including pre-genomic RNA, cccDNA serves as a stable, extra-chromosomal transcriptional template.[6]

Quantitation of intrahepatic cccDNA is proposed to be a useful marker in evaluating the cure of CHB and measuring treatment endpoints.[7] The amount and transcriptional activity of cccDNA in the hepatocytes is critical for HBV disease progression and clinical outcomes. The main barrier to cccDNA quantification, however, is the invasiveness of liver biopsy, which greatly limits the utility of cccDNA as a marker in actual clinical practice. Finding noninvasive surrogate indicators of intrahepatic cccDNA is therefore clinically significant. The intrahepatic cccDNA profile has been predicted to be reflected by a number of conventional serum indicators, including quantitative HBsAg and HBV DNA pregenomicRNA.[8,9] All viral RNAs, including HBV messenger RNAs (mRNAs) and pgRNAs, are produced by cccDNA using cellular transcriptional machinery. These RNAs are crucial for protein synthesis and viral replication.[10] Despite playing a minor part in the prediction of HBsAg loss, HBV RNA is a powerful HBeAg seroconversion monitor.[11]

Material and Methods

Up to 89 patients with persistent HBV infection participated in a cross-sectional study at the GIT hospital in Baghdad's Medical City. The patients for recruitment must be older than 18 years old with CHBV without prior or current anti-HBV treatment and patients will taking antiviral treatment from 3 to 6 months of therapy and their liver fibrosis were monitored by fibroscan excluded patients have other chronic disease or patients on immunosuppressive or immune-modulating therapy for any reason during a period from the first of September 2022 to the end of January 2023.

Nucleic acid extraction from plasma

Nucleic acids were extracted by using a viral nucleic acid extraction kit (Geneaid cat. no. VR100) using the total NA protocol and 200 µL of plasma as input volume and 50 µL elusion volume (as manufactures protocol).

Determination of concentration and purity of DNA and RNA

Concentration and purity were determined by the nanodrop system. Two microliters of DNA and RNA were placed in the machine socket, and DNA and RNA concentration were determined with the OD 260/280 nm, whereas the purity was quantified by using the wavelength 260/230 nm. A ratio of ~2.0 is generally accepted as "pure" DNA and ~1.8 is generally accepted as "pure" for RNA.[12]

Quantification of HBV DNA and HBV RNA in plasma

For the detection of HBV RNA and DNA in plasma, total nucleic acid was isolated from plasma and separated into two tubes: the first one was treated with DNAse1 to degrade DNA, and the remaining RNA was reverse transcribed by BIONEER Accupower RocketScript RTPreMIX (Korea) and HBV-random hexamer primer (Dn12), Oligo(dT) primer, and the reaction was performed under the following conditions.

Primers annealing: 15°C for 10 min, cDNA synthesis: 60°C for 1 h. Heat inactivation: 95°C for 5 min. DNA was treated with RNAse enzyme to degrade RNA. Real-time PCR assay targeting a conserved segment of the core region was used to quantify HBV DNA and HBV RNA in parallel. Amplifications were performed in an Agilent technologies Stratagene MX3005P, using forward primer, GGTCCCCTAGAAGAAGAACTCCCT (nt 2367–2390), reverse primer, CATTGAGATTCCCGAGATTGAGAT (nt 2454–2431),[13] and DNA and RNA amplification was carried out in 20 µL reaction mixtures containing 10 µL universal PCR master mix (perfect start GqPCR SUPER MIX, TRAN, China) for DNA, RNA, and 1 µL of each primer and 3 µL D.D water, and 5 µL of extracted nucleic acids (94°C, 1 min) denaturation steps were followed by 40 cycles of amplification (94°C, 10 s, 58°C, 20 s, 72°C, 25 s).

Agarose gel electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplified bands and to make positive control for the standard curve to obtain results [Figure 1], ethidium bromide-stained bands in gel were visualized to UV using a UV trans-illuminator (TFX-35M), and then photos were taken using a mobile device camera (Realme, Taiwan) (size of DNA is 88bp) then liquefaction the band by Gel/PCR DNA fragments extraction kit (cat no. DF100).

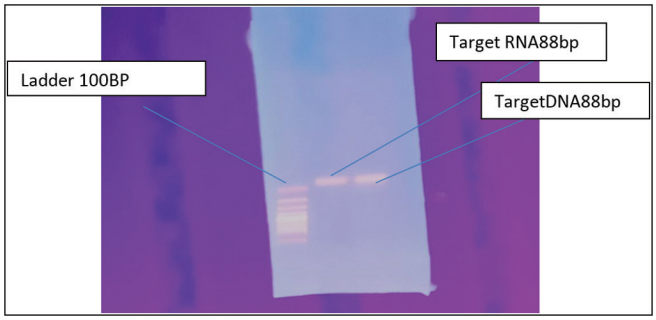

Figure 1: Gel electrophoresis

Standard curve analysis

The electrophoretic bands were extracted from the gel and the resultant DNA concentration was read by nanodrop and then, an online calculator was used to determine the number of copies of the amplicon this calculator requires the user to input the amount of templates present (in ng) and the length of template (in bp) to obtain copy number and create known concentration standards.

Determination of HBeAg status

To determine HBe Ag status we used the ELISA kit (Monocent, Inc., USA) qualitative method. Samples with an OD value lower than the cutoff were classified as negative for HBeAg, and samples with an OD value higher than the cutoff were classified as positive for HBeAg (cutoff = 0.119). Sensitivity and specificity were 99.5% and 99.4%, respectively.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with the patient's verbal and analytical approval before the sample was taken. The study protocol, the subject information, and the consent form were reviewed and approved by a local ethics committee according to document number 112 (including the number and the date on May 11, 2022) to get this approval.

Results

Study group distribution

A total of 89 CHB patients were collected from GIT hospital in the medical city in Baghdad (52 male and 37 female) with age range 18–80 years had undergone fibro scan staging 42 (47.2%F0), 20 (22.5%F1), 11 (12.4%F2), 11 (12.4%F3), and 5 (5.6%F4) as shown in Table 1.

Regarding Hbe Ag status, 94.4% were negative and 5.6% were positive.

Regarding the presence of fibrosis and severity of fibrosis, the percentages are shown in Table 2.

The findings on the association between severity of fibrosis and positivity of HBeAg indicated that positive

Table 1: Frequency of staging

Grade of fibrosis	Frequency	%	Cumulative %
F0	42	47.2	47.2
F1	20	22.5	69.7
F2	11	12.4	82.0
F3	11	12.4	94.4
F4	5	5.6	100.0
Total	89	100.0	

Table 2: Frequency of severity of fibrosis

Severity of fibrosis	Frequency	%	Cumulative %
No fibrosis	42	47.2	47.2
Mild-moderate (F1-F2)	31	34.8	82.0
Severe-advanced (F3-F4)	16	18.0	100.0
Total	89	100.0	

HBeAg was a significant association with severe fibrosis ($P < 0.05$) as shown in Table 3 and Figure 2.

Table 4 and Figure 3 show that HBeAg was associated with the staging of fibrosis ($P \leq 0.01$). About 41 (97.6%) F0 were HBeAg negative and 1 (2.4%) F0 were HBeAg positive, about 20 (100%) F1 were HBeAg negative, 0% were HBeAg positive. About 10 (90.9%) F2 were HBeAg negative, 1 (9.1%) F2 was HBeAg positive, about 8 (72.7%) F3 was HBeAg negative, 3 (27.3%) F2 were HBeAg positive, and 5 (100%) F4 were HBeAg negative, (0%) F4 were HBeAg positive.

It was found that pgRNA level was intermediate directly correlated and significantly associated with DNA levels ($R = 0.31$, $P = 0.01$), weakly directly correlated and significantly associated with ALT ($R = 0.21$, $P = 0.05$), and intermediate directly correlated and significantly associated with AST ($R = 0.25$, $P = 0.01$), and intermediate directly correlated, and significantly associated with age ($R = 0.27$, $P = 0.01$) as shown in Table 5.

The study showed a significant positive correlation between RNA and DNA levels among studied patients as shown in Figure 4.

ROC curve analysis for F3 stage of fibrosis

ROC curve analysis was applied to pgRNA levels in CHB patients with stage 3 of fibrosis (severe fibrosis). It was found that cutoff value of pgRNA (1119.35 copies/ml) and above can predict the presence of severe fibrosis in the liver (sensitivity 81%, specificity 70%).

ROC Curve analysis for F4 stage of fibrosis

ROC Curve analysis was applied to pgRNA levels in CHB patients with stage 4 of fibrosis(advanced fibrosis). It was found that cutoff value of pgRNA (2420.77 copies/mL) and above can predict the presence of severe fibrosis in the liver (sensitivity 80%, specificity 88%).

Table 3: Association between Severity of fibrosis and HBeAg

$P = 0.041$			HBeAg		Total
			Negative	Positive	
Severity of fibrosis	No fibrosis	Count	41	1	42
		% within the severity of fibrosis	97.6%	2.4%	100.0%
		% within HBeAg	48.8%	20.0%	47.2%
		% of total	46.1%	1.1%	47.2%
	Mild-moderate (F1-F2)	Count	30	1	31
		% within the severity of fibrosis	96.8%	3.2%	100.0%
		% within HBeAg	35.7%	20.0%	34.8%
		% of total	33.7%	1.1%	34.8%
	Severe-advanced (F3-F4)	Count	13	3	16
		% within severity of fibrosis	81.3%	18.8%	100.0%
		% within HBeAg	15.5%	60.0%	18.0%
		% of total	14.6%	3.4%	18.0%
Total		Count	84	5	89
		% within severity of fibrosis	94.4%	5.6%	100.0%
		% within HBeAg	100.0%	100.0%	100.0%
		% of total	94.4%	5.6%	100.0%

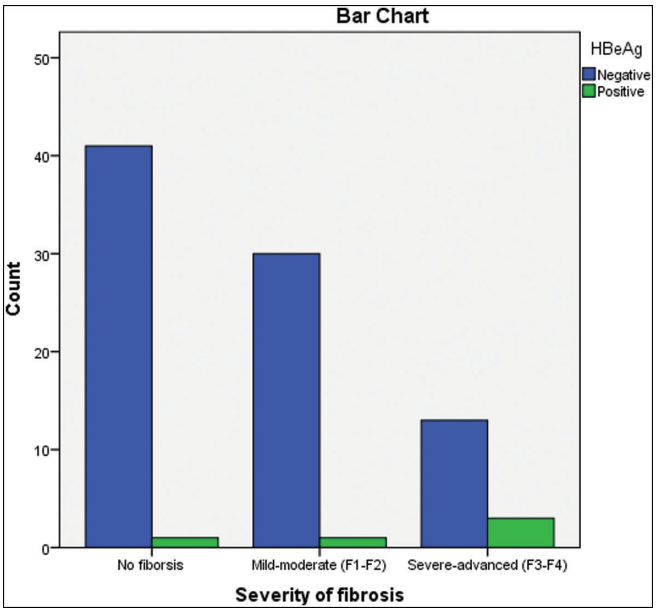

Figure 2: Bar chart HBeAg, the severity of fibrosis in groups in terms of the association between severity of fibrosis × HBeAg

Comparison of the level of DNA and pgRNA of HBV in CHB patients with stage of fibrosis indicated a clear trend of increased level of pgRNA compared to DNA of HBV with more severe stages of liver fibrosis as shown in Figure 5.

Discussion

The persistent HBV infection continues to have an impact on millions of people worldwide. The incidence of chronic HBV was 0.7% in Babylon governorate; hepatitis B is an endemic disease in Iraq.[14] HBV cccDNA is the template for five viral transcripts: preC/core RNA, pgRNA, preS1 RNA, preS2/S RNA, and X RNA.[15]The HBV core promoter (CP) regulates the synthesis of two overlapping 3.5 kb viral transcripts.[16,17] The longer preC mRNA is translated to produce the preC protein, which is the precursor to the HBe antigen (HBeAg).[18] pgRNA not only is the direct transcription product of cccDNA but also can encode HBV polymerase which can convert pgRNA into rcDNA. The rcDNA can be then repaired to form HBV cccDNA.[5] In this study, our goal is to evaluate the quantity of HBV pgRNA. The quantity of HBV cccDNA in liver tissue has a direct correlation with liver fibrosis; hence this study aims to assess the correlation between novel markers pgRNA, and conventional biomarkers namely viral genomic DNA, and qualitative HBsAg profile. Moreover, the current study compared the level of HBV pgRNA with that of HBeAg status (positive or negative) in chronic hepatitis B (CHB) patients In this study, we have analyzed HBV RNA in serum from patients with chronic infection with the virus. An intermediate direct correlation between viral RNA and DNA was identified ($R = 0.31$, $P < 0.01$, $R^2 = 0.91$). These results agreed with a study done by Prakash *et al.*,[13] which reported that serum levels of HBV DNA and HBV RNA correlated with a Spearman's rho of 0.93, an intermediate correlation between DNA and RNA may be influenced by mutation. The serum levels of HBV RNA in our study were almost as high as HBV DNA levels. DNA we saw in our study is reliable because we used the same extracted nucleic acid sample for both analyses, which used the same primers for DNA and RNA and were carried out together. To make sure the RNA assay did not amplify DNA, we pretreated the extracted nucleic acids with DNase and ran the PCR assay in parallel. By doing so, we made sure the increased HBV RNA levels were not caused by HBV DNA amplification. The strategy to use the same primers

Table 4: Association between the staging of fibrosis and HBeAg

P = 0.015			HBeAg		Total
			Negative	Positive	
Staging (F0-F4)	F0	Count	41	1	42
		% within staging (F0-F4)	97.6%	2.4%	100.0%
		% within HBeAg	48.8%	20.0%	47.2%
		% of total	46.1%	1.1%	47.2%
	F1	Count	20	0	20
		% within staging (F0-F4)	100.0%	0.0%	100.0%
		% within HBeAg	23.8%	0.0%	22.5%
		% of total	22.5%	0.0%	22.5%
	F2	Count	10	1	11
		% within staging (F0-F4)	90.9%	9.1%	100.0%
		% within HBeAg	11.9%	20.0%	12.4%
		% of total	11.2%	1.1%	12.4%
	F3	Count	8	3	11
		% within staging (F0-F4)	72.7%	27.3%	100.0%
		% within HBeAg	9.5%	60.0%	12.4%
		% of Total	9.0%	3.4%	12.4%
	F4	Count	5	0	5
		% within staging (F0-F4)	100.0%	0.0%	100.0%
		% within HBeAg	6.0%	0.0%	5.6%
		% of total	5.6%	0.0%	5.6%
Total		Count	84	5	89
		% within staging (F0-F4)	94.4%	5.6%	100.0%
		% within HBeAg	100.0%	100.0%	100.0%
		% of total	94.4%	5.6%	100.0%

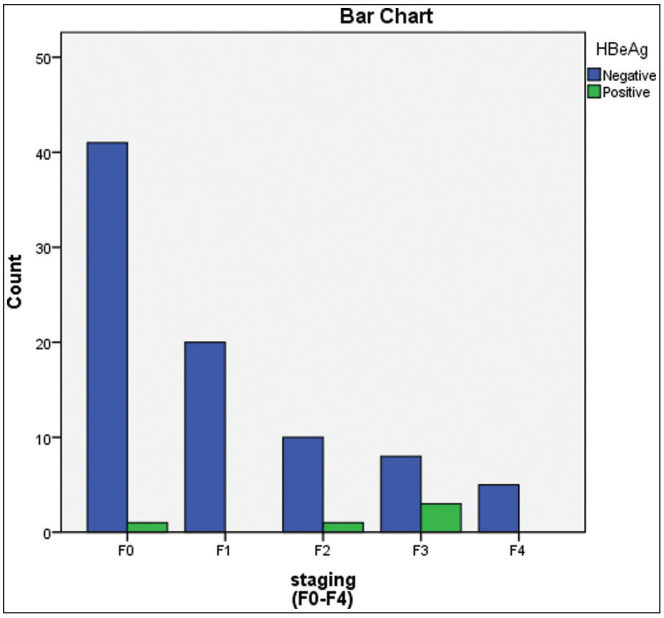

Figure 3: Bar chart on association of HBeAg and staging of fibrosis in CHB patients

and to run DNA and RNA amplifications in parallel probably contributed to the strong correlation. Lin *et al*.[19] studied the relationship between serum pgRNA and HBV cccDNA levels and investigated if serum pgRNA is a reliable marker that represents HBV cccDNA values, serum HBV DNA, and HBsAg. They concluded that HBV pgRNA levels in serum can be a marker that reflects intrahepatic HBV cccDNA compared with serum HBV DNA and HBsAg. This is essential to assess viral replication and its role in liver fibrosis

A study conducted in 2021 by Jaroenlapnopparat *et al*.[20] in Bangkok, Thailand reported more reliable indicators that can assist in safe drug cessation, PgRNA and serum HBcrAg are new virological markers. this prospective study demonstrates the significant role of two novel indicators in patients with chronic HBV infection, especially in predicting VR after drug discontinuation, virological recurrence needs dynamic monitoring of intrahepatic cccDNA levels can predict sustained virological response after cessation of NAs therapy. The application of intrahepatic cccDNA in actual clinical practice has been extremely restricted by the invasiveness of dynamic liver biopsy and the possibility of sampling error. As a result, we must determine the key elements of VR. Several studies have shown that serum HBV pgRNA is associated with viral rebound after withdrawal of treatment.[21-23] Increased HBV RNA levels can also be a marker for viral relapse after NUC discontinuation. In an attempt to find a beneficial marker to be included assessment of liver fibrosis, we performed ROC curve analysis in this study; we observed that serum PgRNA at cutoff values of more than 1119.35 copies/mL RNA can predict the presence of severe fibrosis in the liver (sensitivity 81%, specificity 70%) that means truly positive was 81% only 19% was false positive, 70% specificity means 70% was truly negative only 30% incorrectly identified false positive and when applying on stage 4 of fibrosis (F4 advanced fibrosis) it was found that cutoff value (2420.77 copies/mL) of PgRNA it was above which any level of PgRNA can predict the presence of advanced fibrosis in the liver (sensitivity 80%, specificity 88%) that means 80% was truly positive, 20% was false positive, specificity 88% was truly negative, 12% incorrectly identified as a false positive, positive pgRNA was discovered to have a risk factor of positive HBV DNA. This was in line with the findings of earlier population studies.[19,24] HBV DNA in serum was mostly produced by reverse transcription of HBpgRNA. Additionally discovered to be a risk factor for positive pgRNA was positive HBeAg. This study discovered that age was positively intermediately correlated with PgRNA (R = 0.273, P = 0.01), which may be due to young people's higher immune, more active organ functions, faster liver cell regeneration, and less complications, as well as the fact that age represents

Table 5: The correlations between pgRNA, DNA, ALT, AST, ALP

	DNA (copy/mL)	RNA (copy/mL)	ALT	AST	ALP	Age
DNA (copy n/mL)						
Pearson correlation	1	0.312	0.177	0.103	−0.073	0.014
Sig. (two-tailed)		0.004*	0.105	0.347	0.510	0.897
N	85	85	85	85	85	85
RNA (copy n/ml)						
Pearson correlation	0.312	1	0.211	0.251	0.037	0.273
Sig. (two-tailed)	0.004*	88	0.048*	0.018*	0.732	0.01*
N	85		88	88	88	88
ALT						
Pearson correlation	0.177	0.211	1	0.877	0.112	−0.008
Sig. (two-tailed)	0.105	0.048*	89	0.00*	0.296	0.941
N	85	88		89	89	89
AST						
Pearson Correlation	0.103	0.251	0.877	1	0.169	0.068
Sig. (two-tailed)	0.347	0.018*	0.000*	89	0.113	0.524
N	85	88	89		89	89
ALP						
Pearson correlation	-0.073	0.037	0.112	0.169	1	0.039
Sig. (two-tailed)	0.510	0.732	0.296	0.113	89	0.719
N	85	88	89	89		89
Age						
Pearson correlation	0.014	0.273	−0.008	0.068	0.039	1
Sig. (two-tailed)	0.897	0.01*	0.941	0.524	0.719	89
N	85	88	89	89	89	

An asterisk (*) in the cell gives a significant correlation

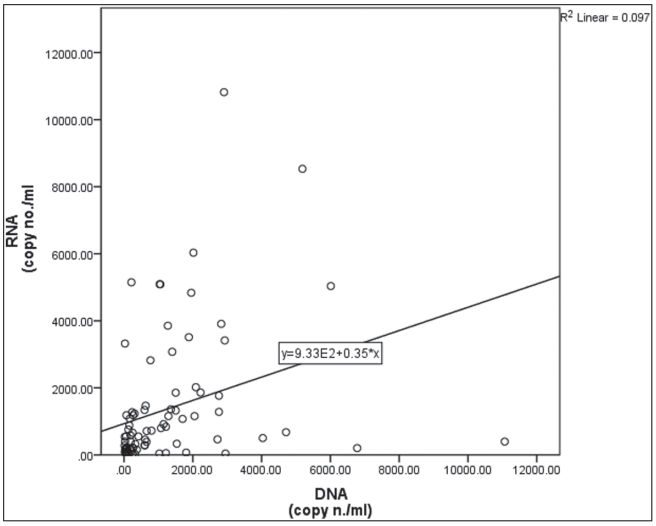

Figure 4: Linear regression analysis shows a clear correlation between DNA level copies/mL and pgRNA copies/mL as pgRNA was dependent on DNA, which means if we need to obtain pgRNA value we can use this equation $Y = 9.33E^2 + 0.35 \times X$ ($R^2 = 0.9$)

disease duration. Briefly, this study defined the status of pgRNA and examined its influencing factors, proved the strong link between pgRNA and HBeAg, as well as HBV DNA, and highlighted the importance of pgRNA in liver fibrosis, enhanced the clinical significance of pgRNA, and suggested future directions. Additionally, this study examined the age effect and the factors that affected the amplitude of pgRNA changes, both of which had a certain guiding relevance for clinical medicine. There is no disputing that this study also had a number of shortcomings. Because it was conducted at a single hospital, this study's cross-sectional design made it difficult to accurately reflect the total community. Furthermore, Prakash *et al*.[25] in 2020 discovered that the predominant HBV RNA present in the serum of patients with chronic hepatitis B was pgRNA rather than pre-C mRNA. pgRNA made up the majority of the HBV RNA in serum, however, pre-C RNA is a hairpin longer than pgRNA.[25] Designing specialized primers for pgRNA was made difficult by this. Genotype differences were not compared due to the lack of genotypic data, but all patients were Iraqis; therefore, they were most likely infected with type D. Studies conclude that pgRNA enhanced the clinical significance and suggested the future directions, so it is a promising biomarker to predict liver fibrosis. It is a surrogate biomarker for cccDNA, which is a noninvasive, cost-effective method; recommends an increase in sample size and studies of different genotypes should also be analyzed. Age effect and the factors that affected the amplitude of pgRNA changes, such as HBeAg status both of which had a certain guiding relevance for clinical medicine pgRNA can be used in the management

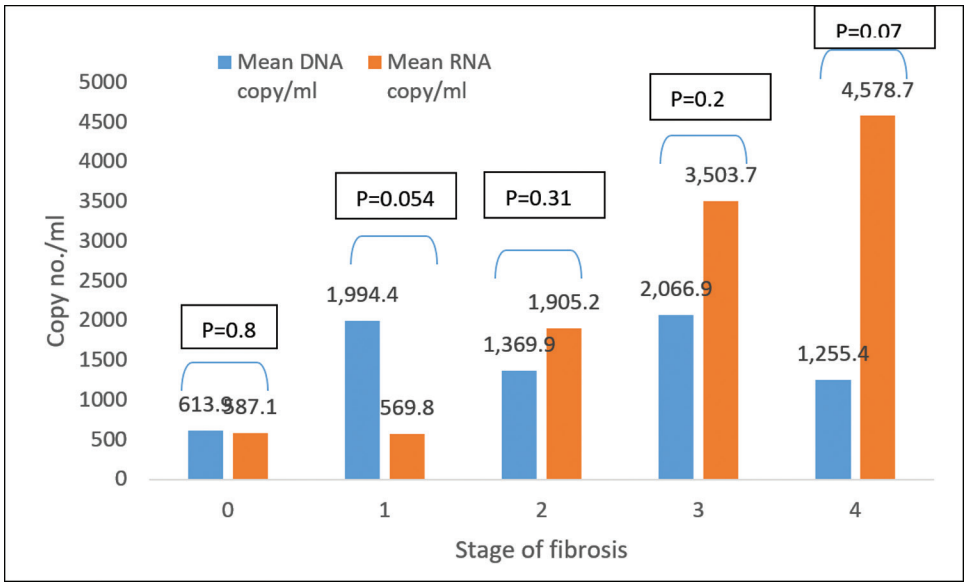

Figure 5: Comparison of the mean level of pre-genomic RNA versus DNA in CHB at different stages of liver fibrosis with *P* value calculated from independent *t* tests

of CHB patients and predict recovery from disease and discontinuing the treatment recommendations increase sample size further studies to understand the mechanism of failing reverse transcription, genotyping.

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Conflicts of interest

There are no conflicts of interest.

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