A Study on Pregenomic RNA and Factors Related to Hepatitis B Virus Infection Based on Real World

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Research

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Abstract

Objective: To study the clinical significance and influencing factors of pgRNA based on real world.

Methods: A total of 421 patients who were tested for pgRNA were selected. According to the baseline data, the subjects were divided into negative and positive group. Chi-square test and logistic regression were used to analyze the influencing factors of pgRNA status. Based on the follow-up data, rank sum test and linear regression were used to analyze the influencing factors of pgRNA change magnitude.

Results: 153 (36.3%) of the 421 subjects were pgRNA negative and 268 (63.7%) were pgRNA positive. Logistic regression analysis showed that positive HBV DNA (OR: 40.51), positive HBeAg (OR: 66.24), tenofovir treatment (OR: 23.47) and entecavir treatment (OR: 14.90) were independent risk factors for positive pgRNA. Univariate linear regression showed that pgRNA change magnitude of patients treated with entecavir was higher than that of patients treated with tenofovir. Multivariate linear regression showed that age was an independent factor influencing pgRNA change magnitude.

Conclusions: The pgRNA of patients who were young, female, HBV DNA-positive, high-HBsAg, HBeAg-positive is higher than the detection line. HBV DNA, HBeAg are independent risk factors for positive pgRNA. Different antiviral regimens have significantly different effects on pgRNA status. There was a significant correlation between pgRNA and FIB-4, suggesting that pgRNA is related to liver fibrosis. The decrease in pgRNA was greater in young patients than in non-young patients. The decrease in pgRNA was greater in patients treated with tenofovir than in patients treated with entecavir.

Introduction

HBV (hepatitis B virus), as a kind of global communicable disease, is prevalent in Asian and African countries especially. There are approximately 257 million chronic HBV infections in the world. It is estimated that there are currently about 70 million chronic HBV infections in China, including about 20 to 30 million CHB (Chronic Hepatitis B) patients[1].

It[2, 3] is found that during the life circle of hepatitis B virus, HBV cccDNA (cova-lently closed circular DNA) in liver cells, as the only template of all the HBV RNA coding, is not only the begining of virus replication, but also the key factor of persistent infection and recurrence. The current therapies are proved to exert little influence on cccDNA, the long-term existence of which is an important reason of chronic HBV[4]. Theoretically, cccDNA level should be the most direct index of prognostic[5]. However, due to[6, 7, 8] its uneven distribution and damage caused by invasive examination, the application of cccDNA is limited. Conversely, the examination of HBV pgRNA (pregenomic RNA) is simple and non-invasive for the sampling, thus more acceptable to patients[9].

It[4] is proved that pgRNA is not only the direct transcription product of cccDNA and the template of HBV polymerase, but also can be retrotranscribed into rcDNA (relaxed circular DNA) and then converted back

to HBV cccDNA. The research[10] also suggests pgRNA is positively correlated with cccDNA. It's obvious that pgRNA have chance to replace cccDNA to play the key role in antiviral therapy.

The current evaluation of antiviral therapy efficacy depends on HBV DNA[11, 12]. However, after the reverse transcription of pgRNA is controlled, the serum HBV DNA levels of CHB decrease, while the levels of cccDNA decrease little. Below-detection-limit HBV DNA only means the reverse transcription is restrained, instead of the indicator of withdrawal, because cccDNA may still express[11, 12]. Theoretically, even one replicate-capable cccDNA in liver cells can lead to recurrence once antiviral therapy is stopped[11]. Therefore, the removal of cccDNA is considered important to eradicate persistent HBV infection[3]. AASLD (American Association for the Study of Liver Diseases) and EASL (European Association for the Study of the Liver) also defined "HBV completely cured" as "HBsAg (Hepatitis B surface Antigen) in serum is undetectable and HBV DNA is eradicated, including eradication of cccDNA and integrated HBV DNA in liver." Therefore, apart from HBV DNA, it is necessary to follow cccDNA to estimate the prognosis of HBV patients.

Furthermore, nucleos(t)ide drugs can only block the reverse transcription of pgRNA without affecting its production[13, 14]. Even when HBV DNA synthesis is blocked, cccDNA can still synthesize new HBV particles in the HBV RNA virus-like particle mode[15]. Therefore, when HBV RNA cannot be detected in serum, cccDNA in liver has disappeared or stopped transcribing, which means the medication can be stopped[10, 16].

It is necessary to conduct epidemiological studies on pgRNA, since not many those have been published currently.

Objects And Methods

Patients and Data Collection

Eligible participants were adults with confirmed hepatitis B virus infection and complete case-related information, who have detected pgRNA at the Fifth Affiliated Hospital of Guangzhou Medical University from 2019.8.23 to 2021.1.23. The exclusion criteria were the following: co-infection with HIV (Human Immunodeficiency Virus), syphilis or other viral hepatitis such as HCV (Hepatitis C Virus), HDV (Hepatitis D Virus) and so on.

Totally 421individuals were selected, whose relevant data in the inspection system were collected, including pgRNA, HBV DNA, HBsAg, HBeAg (Hepatitis B e Antigen), ALT (Alanine Aminotransferase), AST (Aspartate Aminotransferase), PLT (Platelet Count), etc. Through the medical record management system, the information about basic characteristic and treatment were collected, such as age, gender, treatment plan, etc. The patient information involved has been replaced by initials and medical record numbers, and would not involve patient privacy.

The baseline data was defined as the data when pgRNA was first detected within the study period. The data measured at the first follow-up after the baseline was retrospectively collected to conduct a retrospective cohort study.

Detection Of Pgrna

pgRNA were detected by Hepatitis B Virus pgRNA Detection Kit (detection limit: 200 Copies/mL, PCR (Polymerase Chain Reaction)-Fluorescent Probing, Guangzhou SUPBIO Biotechnology Co., Ltd., Guangdong, China), which used HBV specific primers and probes (Taqman probe), with PCR reaction solution, thermostable DNA polymerase (Taq enzyme), reverse transcriptase (RT enzyme), RNase inhibitor, deoxynucleus triphosphates (dNTPs) and other components. This kit was amplified by RT-PCR and used for the quantitative detection of HBV pgRNA in the sample. Besides, It set up an internal control and by detecting whether the internal control was normal, it could exam whether there were false negatives caused by factors such as reagents, PCR inhibitors and so on.

Statistical Analysis

The statistical analyses were performed by IBM SPSS statistics (version 25.0). Continuous variables were found non-normal by one-sample Kolmogorov-Smirnov test and expressed as medians (inter-quartile ranges). Categorical variables were expressed as frequency (percentage). The variables were compared between groups using Mann-Whitney U and Chi-square test for univariate comparisons as well as the Kruskall-Wallis H test for multivariate comparisons. Logistic regression analysis was used to explore the influencing factors of pgRNA status. Linear regression analysis was used to explore the influencing factors of the change magnitude of pgRNA and HBsAg. Statistical significance was defined as $P < 0.05$ (two-tailed).

Results

Exploration about the related factors of pgRNA status based on baseline data

Compared with the pgRNA-negative, the pgRNA-positive had statistically higher composition ratios of factors, including youth (85.8% VS 65.4%), female (47.4% VS 35.3%), positive HBV DNA (73.1% VS 39.0%), high HBsAg-level (60.4% VS 38.8%), and positive HBeAg (76.1% VS 3.6%). The compositions of the previous treatment plan before baseline were also statistically different between different pgRNA groups, so were those of FIB-4 grade ($P < 0.05$) (Table 1).
Tenofovir treatment (OR: 23.47, 95%CI: 3.35 ~ 164.65) was an independent risky factor for positive pgRNA, so were entecavir treatment (OR: 14.90, 95%CI: 2.18 ~ 101.90), positive HBV DNA (OR: 40.51, 95%CI: 7.00 ~ 234.55) and positive HBeAg (OR: 66.24, 95%CI: 20.03 ~ 219.07) ($P < 0.05$). Conversely, there

were no statistical significance of factors, including age (OR: 1.14, 95%CI: 0.46 ~ 2.81), gender (OR: 1.43, 95%CI: 0.64 ~ 3.19) and other treatment plans (OR: 3.81, 95%CI: 0.46 ~ 31.54) ($P > 0.05$) (Table 2).

Exploration about the clinical significance between pgRNA and liver fibrosis on baseline data

As mentioned above, the compositions of FIB-4 grade were statistically different between different pgRNA groups ($P < 0.05$) (Table 1). The number of patients with FIB-4 below grade 2, grade 2 and above grade 2 were 73, 7, and 4 respectively in the baseline HBV DNA-negative group, and 105, 7 and 7 respectively in the baseline HBV DNA-positive group. As shown in Fig. 1(A), there was no statistical difference between different HBV DNA groups ($\chi^2 = 0.553$, $P = 0.758$).

As shown in Fig. 1(B), in the baseline, the M (P25, P75) of FIB-4 index was 0.68 (0.40, 1.11). The M (P25, P75) of FIB-4 index of pgRNA-negative group was 0.76 (0.47, 1.45), and that of pgRNA-positive group was 0.58 (0.33, 0.91). The difference was statistically significant (W = 21019, $P = 0.001$). Conversely, the M (P25, P75) of FIB-4 index of HBV DNA-negative group was 0.68 (0.49, 0.88), and that of HBV DNA-positive group was 0.60 (0.38, 1.07). The difference was not statistically significant (W = 11709, $P = 0.298$).

Obviously, whether FIB-4 grades or FIB-4 indexes were statistically different between different pgRNA groups, while not statistically different between different HBV DNA groups, suggesting pgRNA had a better correlation with liver fibrosis than HBV DNA.

Exploration about the influencing factors of pgRNA change magnitude based on retrospective cohort study

At last, totally 89 patients who had detected pgRNA twice or more times were included, among which 20 (22.5%) were negative for baseline pgRNA. Among the remaining 69 (77.5%), 7 (10.1%) were pgRNA-negative in the second detection. When calculating pgRNA change magnitude, the amounts of pgRNA for the second detection were estimated at 100 Copies/mL for the seven people who were negative in the second detection. The change magnitude of pgRNA was defined as the numerical value of the second detection amount minus the baseline detection amount then divided by the baseline detection amount, so when the magnitude of change was negative, the greater the absolute value, the greater the decline of pgRNA.

There was significant difference of pgRNA change magnitude between different age groups, so was there among different treatment plan groups ($P < 0.05$). However, there was no significant difference of pgRNA change magnitude between different groups of other factors, including gender, treatment time, baseline HBV DNA-status, baseline HBsAg-status, baseline HBeAg-status, FIB-4 grade, and history of formal antiviral therapy ($P > 0.05$) (Fig. 2).

Univariate linear regression about treatment plan showed compared with the patients treated with tenofovir, the patients treated with entecavir (B: 1.806, 95%CI: 0.777 ~ 2.835) had higher pgRNA change

magnitude. The conclusion could be drawn that pgRNA decline of patients treated with tenofovir was greater than that of patients treated with entecavir. There was no statistical significance of other treatment plans (B: -0.285, 95%CI: -1.514 ~ 0.944) (Table 3).

Multivariate linear regression showed age (B: 1.588, 95%CI: 0.268 ~ 2.907) was an independent factor in pgRNA change magnitude. In other words, compared with young patients, non-young patients had higher pgRNA change magnitude. Therefore, pgRNA decline of young patients was greater than that of non-young patients. The multivariate linear regression model showed no statistically significant relationship of gender or treatment plan ($P > 0.05$) (Table 3).

Exploration about the influence of pgRNA on HBsAg change magnitude based on retrospective cohort study

At last, totally 133 patients who had baseline pgRNA data and had detected HBsAg twice or more were included. As shown in Fig. 1(C), if one logarithmic value of HBsAg drop was defined as “HBsAg significantly decreased”, among the baseline pgRNA-positive persons, 3 persons (2.9%) had a significant decrease in HBsAg, so did 1 person (3.4%) among the baseline pgRNA-negative persons. There was no statistical difference between different pgRNA groups ($\chi^2 = 0.025$, $P = 0.875$).

As shown in Fig. 1(D), the M (P25, P75) of HBsAg change magnitude of baseline pgRNA-positive group was − 10.63% (-37.80%, 5.78%), and that of baseline pgRNA-negative group was − 15.19% (-36.62%, -5.08%). There was no statistical difference between different pgRNA groups (W = 1759, $P = 0.316$).

The conclusion could be drawn that no statistical difference was found between different baseline pgRNA groups by calculating whether incidence of significant decrease in HBsAg or HBsAg change magnitude. In other words, the status of baseline pgRNA was not statistically related to the change of HBsAg during treatment.

Discussion

CHB is still one of the major infectious diseases in the world. Previous studies on HBV pgRNA mainly focus on molecular level, while not many large-scale studies focus on public level.

Positive HBV DNA was found a risky factor of positive pgRNA. This was consistent with the conclusions drawn by other population studies[17, 18]. From a molecular perspective, HBV DNA in serum was mainly derived from the reverse transcription of HBV pgRNA[4, 19] (Fig. 3), which could also support this conclusion.

Positive HBeAg was also found a risky factor of positive pgRNA. This was the same conclusion reached by other population studies[4, 20, 21]. From a molecular perspective, HBeAg was derived from pre-C mRNA which was transcribed from HBV cccDNA. Like pgRNA, pre-C mRNA was one of the manifestation of 3.5kb RNA transcribed from HBV cccDNA [22] (Fig. 3). It was speculated that the two kinds of 3.5kb RNA

were transformed according to a certain ratio. In other words, there was a high load of HBV cccDNA and a high degree of HBV virus expression in the liver of patients in the active phase.

This study suggested that patients who had had received regular antiviral therapy with tenofovir or entecavir before detection were more likely to have positive pgRNA than patients who had not had received regular antiviral therapy. This was consistent with the conclusion of this study[23], which showed that HBV pgRNA transcribed with HBV cccDNA as a template would be degraded during reverse transcription, while NAs (Nucleos(t)ide Analogue) drugs could inhibit the reverse transcription process, resulting in the accumulation and increase of HBV pgRNA in the serum of patients receiving NAs treatment (Fig. 3). In addition, one of the possible reasons was that the cross-sectional study cannot correctly identify the defects of causal timing. According to the guidelines of prevention and treatment for the chronic hepatitis B (2019 version)[1], clinical indications for chronic hepatitis B patients were generally based on positive HBV DNA. This study have found that there was a significant correlation between HBV DNA and pgRNA. Therefore, patients who had positive pgRNA were more likely to get positive HBV DNA and then receive antiviral therapy. This study did not observe the significant significance of using other antiviral treatments, which might be related to insufficient sample size.

This study found that FIB−4 grade and FIB−4 index were significantly related to pgRNA status, but not to HBV DNA. This conclusion did not only further validate the relationship between pgRNA and HBV cccDNA in liver cells and the clinical significance of HBV cccDNA in the fibrosis caused by continuous and repeated infection of the liver, but also reminded us that in clinical work, especially for liver fibrosis, in addition to paying attention to HBV traditional indicators such as HBV DNA, we should also focus on HBV pgRNA.

This study found age was an independent factor of pgRNA change magnitude. Young patients had a greater decline in pgRNA than non-young patients. This might be related to young people's stronger immunity, more active organ functions, better liver cell regeneration, and fewer complications, etc.

The univariate linear regression found that patients treated with tenofovir were more likely to get pgRNA decrease than patients treated with entecavir. And the *P* value was also less than 0.1 in the multivariate regression. This suggested that tenofovir's ability to reduce pgRNA may be more superior than entecavir's. In other words, compared with entecavir, tenofovir treatment may make the HBV cccDNA in the patient's liver decline faster and hepatitis B recurrence less likely.

We did not find any significant correlation between pgRNA and HBsAg by calculating whether the categorical variable "whether HBsAg significantly decreased" or the quantitative variable "HBsAg change magnitude". This was consistent with the results in the cross section of this study. However, due to the small sample size and short follow-up time, the possibility of false negatives cannot be ruled out. In addition, referring to the definition of "low viral load" in the study[25] (positive HBsAg but negative HBeAg) and the guidelines[1] (HBV DNA ≤ 2000IU/ml), in this retrospective cohort study, 50 (37.6%) patients were HBeAg-negative, and 62 (46.6%) patients had HBV DNA lower than 2000IU/ml, who could be classified as

patients with low viral load. Studies[10, 26] had found that when the virus in patients was in a state of low expression, a considerable proportion of HBsAg was derived from HBV DNA integrated into the chromosome of the host liver cell. At this time, the HBsAg level was less affected by the pgRNA level, and the relationship between HBsAg and pgRNA also disappeared[27] (Fig. 3). The statistical difference might be able to be observed in case with a larger sample and a longer observation period.

In summary, this study described the status of pgRNA and analyzed its influencing factors, verified the significant correlation between pgRNA and HBeAg as well as HBV DNA, pointed out the relevance of pgRNA in liver fibrosis, enriched the clinical significance of pgRNA, and found that antiviral therapy could also affect the status of pgRNA. Besides, this article studied the influencing factors of pgRNA change magnitude, and further explored the influence of age and antiviral treatment plans on pgRNA change magnitude, which had certain guiding significance for clinical medication.

It is undeniable that this study also had some shortcomings. This study was a retrospective study conducted in a single hospital, which could not represent the entire population well. As a result, the extrapolation ability was not strong. If possible, a multi-center prospective cohort study is supposed to be carried out, expanding sample size under strict inclusion criteria and cost-control, in a bid to further explore the ability of pgRNA to guide and predict the disease occurrence, recurrence, patient withdrawal, and so on.

Conclusions

1. The pgRNA of patients who were young, female, HBV DNA-positive, high-HBsAg, HBeAg-positive was higher than the detection line. HBV DNA, HBeAg were independent risk factors for positive pgRNA.
2. The pgRNA statuses of patients with different baseline treatment regimens were statistically different, indicating different antiviral regimens had significantly different effects on pgRNA status.
3. There was a significant correlation between pgRNA and FIB-4, suggesting that pgRNA is related to liver fibrosis. However, no statistical correlation was found between HBV DNA and FIB-4, indicating that in clinical work, especially for liver fibrosis, the clinical significance of pgRNA should be paid more attention to.
4. The decrease in pgRNA was greater in young patients than in non-young patients. The decrease in pgRNA was greater in patients treated with tenofovir than in patients treated with entecavir.

Abbreviations

pgRNA	pregenomic RNA
HBV	Hepatitis B Virus
CHB	Chronic Hepatitis B
cccDNA	cova-lently closed circular DNA
rcDNA	relaxed circular DNA
AASLD	American Association for the Study of Liver Diseases
EASL	European Association for the Study of the Liver
HBsAg	Hepatitis B surface Antigen
HIV	Human Immunodeficiency Virus
HCV	Hepatitis C Virus
HDV	Hepatitis D Virus
HBeAg	Hepatitis B e Antigen
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
PLT	Platelet Count
PCR	Polymerase Chain Reaction
RT	Reverse Transcriptase
dNTPs	deoxynucleus Triphosphates
NAs	Nucleos(t)ide Analogue

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of The Fifth Affiliated Hospital of Guangzhou Medical University. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

H.Y, S.O, T.P and X.G developed the concept of the study. L.Y, C.L and Z.H participated in its design and coordination and helped draft the manuscript. H.Y, T.P and X.G contributed to the acquisition and interpretation of data. S.O provided a critical review and substantially revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Description and difference analysis of factors under different pgRNA statuses

Factors	Total （%）	pgRNA-negative group（%）	pgRNA-positive group（%）	χ^2 value	*P* value
	421 （100）	153（36.3%）	268（63.7）		
Age†				24.067	<0.001
Youth	330 （78.4）	100（65.4）	230（85.8）		
Non-youth	91 （21.6）	53（34.6）	38（14.2）		
Gender				5.812	0.016
Male	240 （57.0）	99（64.7）	141（52.6）		
Female	181 （43.0）	54（35.3）	127（47.4）		
Previous treatment plan‡				20.960	<0.001
None	206 （48.9）	74（48.4）	132（49.3）		
Tenofovir	109 （25.9）	24（15.7）	85（31.7）		
Entecavir	85 （20.2）	45（29.4）	40（14.9）		
Other treatment plans	21（5.0）	10（6.5）	11（4.1）		
HBV DNA				34.870	<0.001
Negative	122 （38.0）	64（61.0）	58（26.9）		
Positive	199 （62.0）	41（39.0）	158（73.1）		
HBsAg§				13.673	<0.001
Low HBsAg-level	149 （47.6）	71（61.2）	78（39.6）		
High HBsAg-level	164 （52.4）	45（38.8）	119（60.4）		
HBeAg				184.104	<0.001
Negative	190 （50.0）	132（96.4）	58（23.9）		

Positive	190 (50.0%)	5 (3.6%)	185 (76.1%)		
FIB-4 grade¶				9.219	0.010
Below grade 2	228 (83.2%)	79 (75.2%)	149 (88.2%)		
Grade 2	23 (8.4%)	11 (10.5%)	12 (7.1%)		
Above grade 2	23 (8.4%)	15 (14.3%)	8 (4.7%)		

†The variable of age was divided into youth group (18~44Y) and non-youth group (>44Y) according to World Health Organization (WHO) standards for age classification. ‡Previous treatment plan was defined as the type of drug that had been taken by patients before the baseline. §HBsAg was divided into low HBsAg-level group and high HBsAg-level group based on the median of the data in this study with 2000IU/mL as the cut-off point. ¶FIB-4 was calculated as AST [IU/L] × age [years] / PLT [10^9/L] / ALT [IU/L]$^{1/2}$ for reflecting liver fibrosis and cirrhosis; Those with FIB-4 less than 1.45 had no obvious liver fibrosis or only liver fibrosis below grade 2; those with FIB-4 greater than 3.25 had liver fibrosis above grade 2.

Table 2. Multivariate logistic regression analysis results of baseline pgRNA influencing factors

Factors	B	Wald value	*P* value	Odds Ratio (95%CI)
Constant	-4.517	12.764	0.001	0.01
Age	0.131	0.080	0.777	1.14 (0.46 2.81)
Gender	0.356	0.753	0.386	1.43 (0.64 3.19)
Previous Treatment Plan		10.715	0.013	
Tenofovir	3.156	10.083	0.001	23.47 (3.35 164.65)
Entecavir	2.702	7.586	0.006	14.90 (2.18 101.90)
Other treatment plans	1.338	1.541	0.215	3.81 (0.46 31.54)
None	Control			
HBV DNA	3.701	17.064	0.001	40.51 (7.00 234.55)
HBeAg	4.193	47.214	0.001	66.24 (20.03 219.07)

Table 3. The results of linear regression about pgRNA change magnitude

Factors	B（95%CI）	Beta	t value	*P* value
univariate linear regression about the influence treatment plan exert on pgRNA change magnitude				
Constant	-0.305（-0.802，0.192）		-1.225	0.225
Tenofovir	Control			
Entecavir	1.806（0.777，2.835）	0.400	3.503	0.001
Other treatment plans	-0.285（-1.514，0.944）	-0.053	-0.463	0.645
Multivariate linear regression about influencing factors of pgRNA change magnitude				
Constant	-2.307（-4.308，-0.306）		-2.303	0.025
Age	1.588（0.268，2.907）	0.320	2.404	0.019
Gender	0.204（-0.592，1.000）	0.056	0.513	0.610
Treatment Plan				
Tenofovir	Control			
Entecavir	1.021（-0.181，2.223）	0.226	1.697	0.094
Other treatment plans	-0.166（-1.366，1.035）	-0.031	-0.276	0.784

Figures

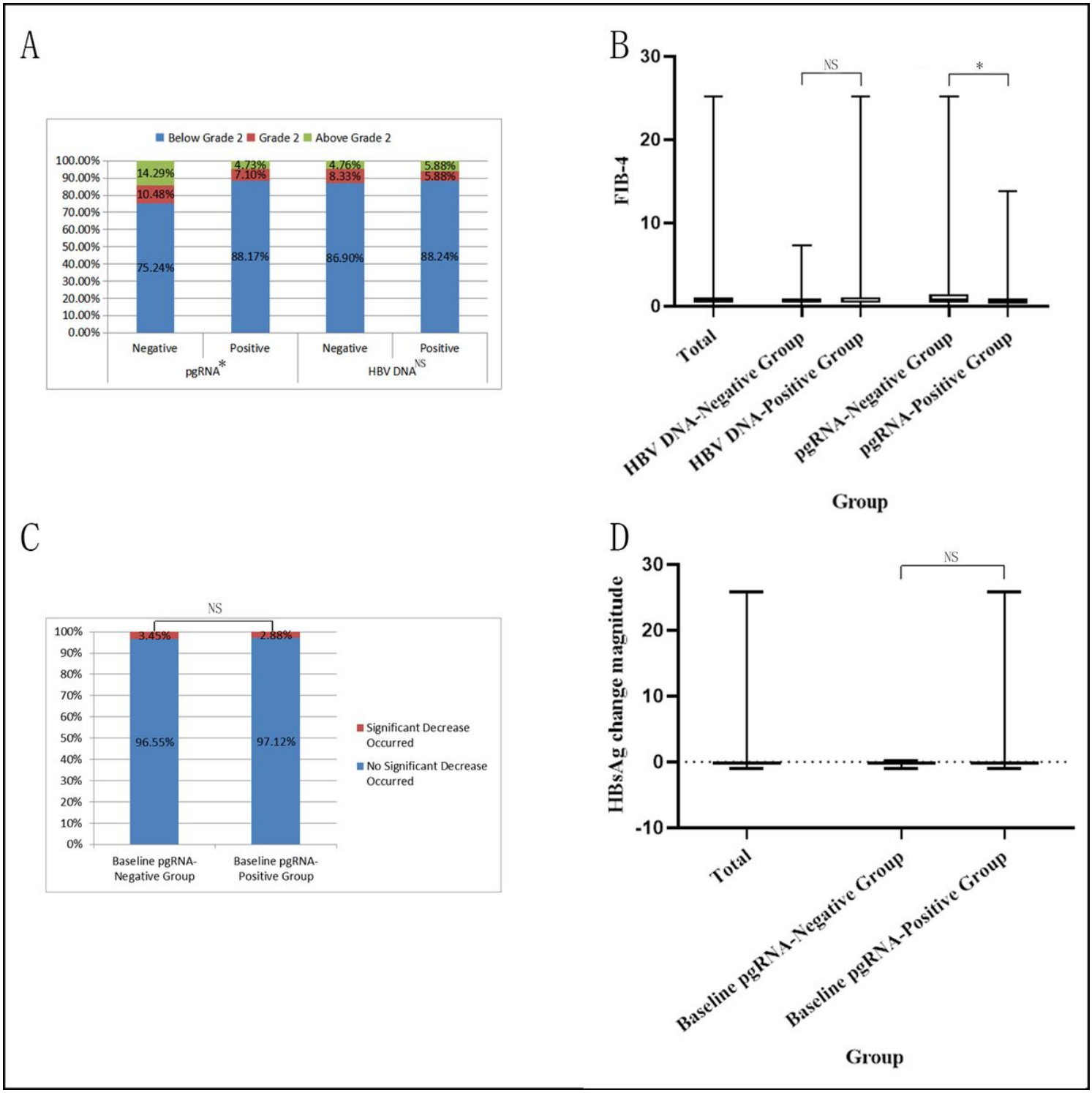

Figure 1

The summary of difference analysis results (A: FIB-4 grade among different baseline pgRNA and HBV DNA groups; B: FIB-4 index among different baseline pgRNA and HBV DNA groups; C: Incidence of significant decrease in HBsAg between different baseline pgRNA groups; D: HBsAg change magnitude between different baseline pgRNA groups) NS: P□0.05; *: P□0.05;

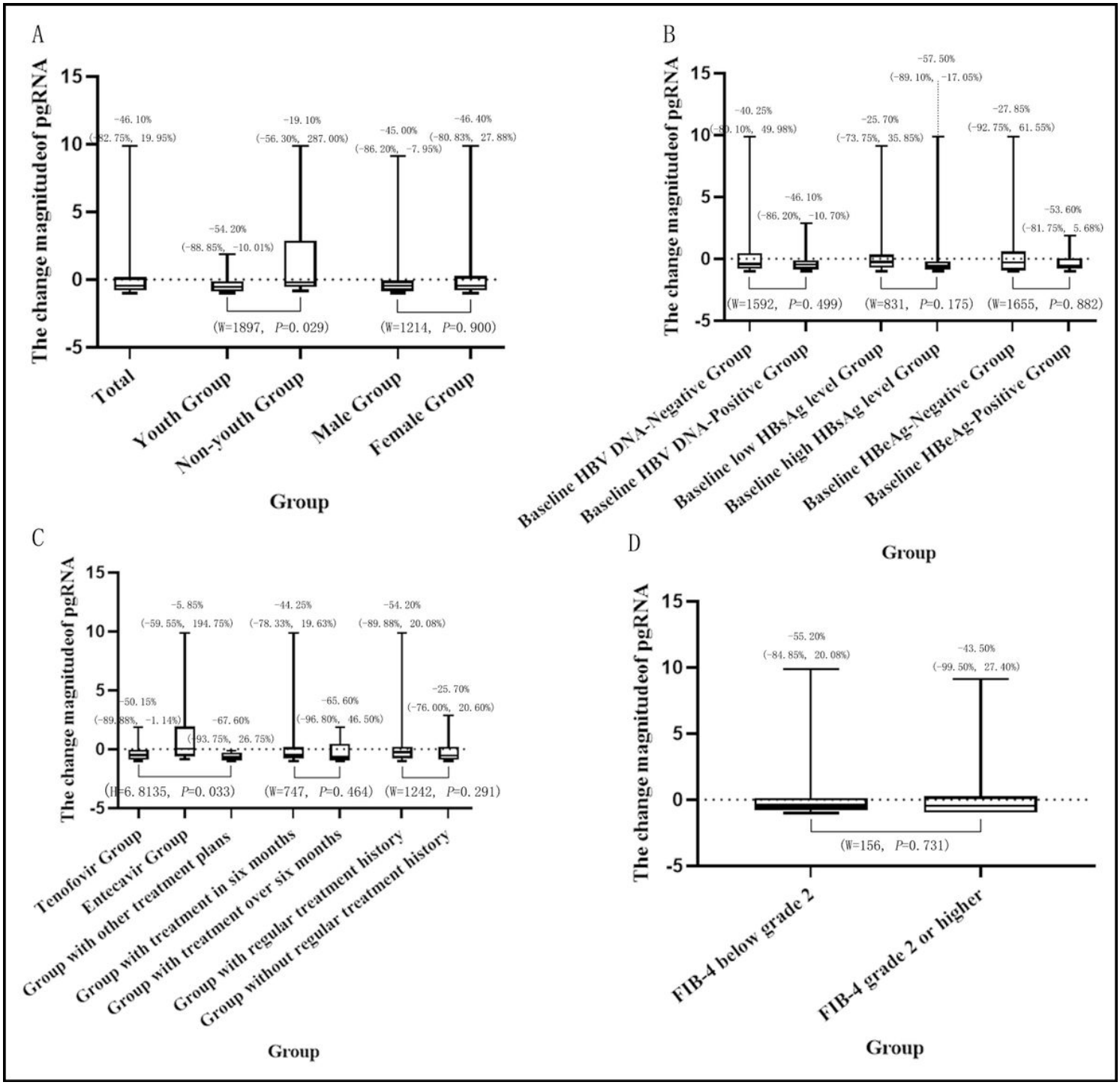

Figure 2

Description and results of difference analysis of the pgRNA change magnitude in different groups (A: General characteristics; B: HBV serum marker status; C: Treatment situation; D: Degree of liver fibrosis)

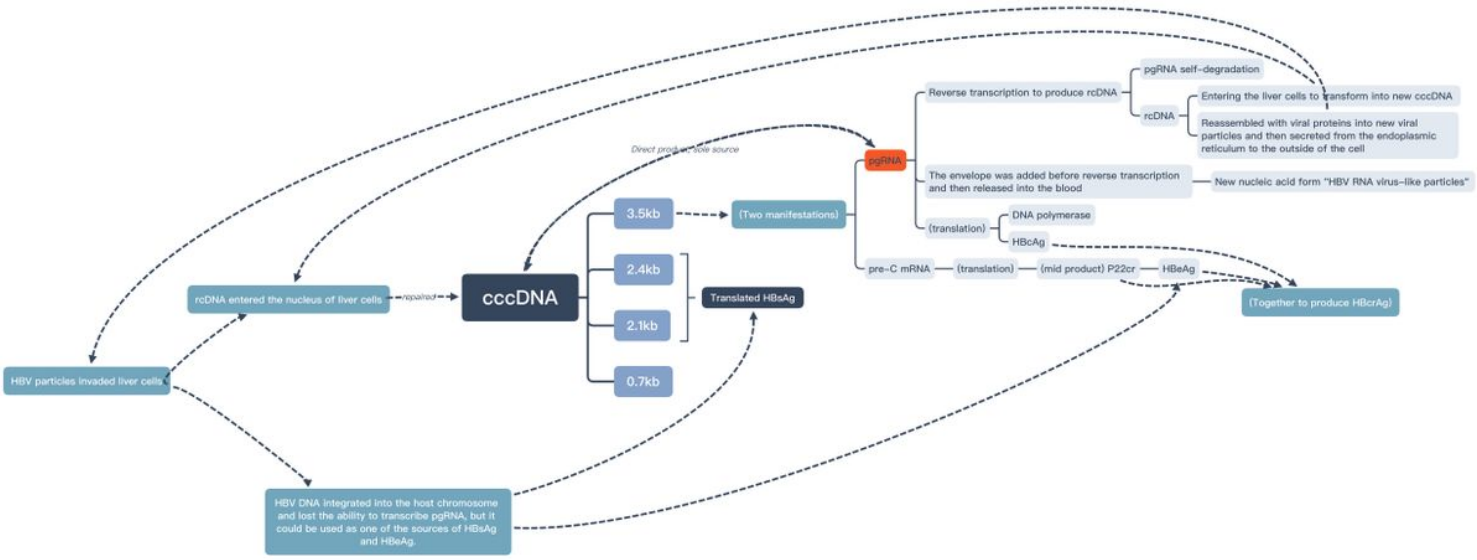

Figure 3

Source and fate of pgRNA and related substances[2, 4, 19, 22, 23, 24]