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ORIGINAL RESEARCH

Nucleos(T)ide Analogue Treatment Has a More Pronounced Impact on Immune Repertoires of CHB Patients Compared to HCC Patients

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Background: Nucleos(t)ide analogues (NAs) as the first-line treatment for chronic hepatitis B (CHB) have been shown to partially restore the antiviral immunity of the patients. However, hepatitis B virus (HBV) related hepatocellular carcinoma (HCC) patients have a relatively longer duration of HBV infection and lower level of HBV DNA. Whether NAs treatments have a different effect on their immune repertoires between CHB and HCC patients remains to be determined.

Patients and Methods: In this study, 126 CHB patients and 85 HBV-related HCC patients who received or did not receive NAs treatment, as well as 361 healthy individuals were enrolled to analyze the effect of NAs treatment on T cell receptor β chain (TCRβ) and B cell receptor heavy chain (BCRh) repertoires in peripheral blood of the patients.

Results: We found that after NAs therapy, the richness and evenness of TCRβ and BCRh repertoires in CHB patients were significantly lower than those in untreated patients and healthy controls, while the diversity of TCRβ and BCRh repertoires was stable in HCC patients. The alanine aminotransferase and HBV DNA levels were not correlated with the TCR or BCR diversity in CHB and HCC patients.

Conclusion: The results suggest that NAs therapy could influence the overall T cell and B cell repertoires diversity in CHB patients but has minimal impact on HCC patients, indicating a significant difference in the potential to restore antiviral immunity between CHB and HCC patients by NAs treatment.

Keywords: chronic hepatitis B, Nucleos(t)ide analogue, T cell receptor, immune repertoire, hepatocellular carcinoma

Introduction

Nucleos(t)ide analogues (NAs) can effectively inhibit hepatitis B virus (HBV) replication, significantly reducing HBV DNA level in chronic hepatitis B (CHB) patients, thereby decreasing the occurrence of liver inflammation and the progression of liver disease. In particular, orally administered NAs are more widely used than interferon-α (IFN-α) due to their convenience and lower cost. However, whether using NAs or IFN-α therapy, complete clearance of HBV is difficult to achieve. NAs or IFN-α alone or in combination treatment achieves hepatitis B surface antigen (HBsAg) seroclearance in less than 10% of CHB patients, and the majority of patients require long-term medication.[1] Hence, the current clinical focus is on functional cure as a treatment goal.

Over 90% of adults with acute HBV infection can control and clear the virus within a short period through their immune response. Apart from direct killing of HBV-infected hepatocytes by $CD8^+$ T cells, a crucial aspect involves the

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cytokines IFN-γ and TNF-α, which, in a proteasome-dependent manner, prevent the assembly of HBV replication intermediates. This process destabilizes the covalently closed circular DNA (cccDNA) in the hepatocellular nucleus, leading to cccDNA clearance without requiring hepatocellular division and without causing cellular damage.[2] However, achieving a cure even with the assistance of antiviral therapy in CHB patients remains challenging. Patients with acute HBV infection generate a vigorous and polyclonal T-cell immune response against multiple epitopes of the virus. The quantity and quality of HBV-specific T cells are higher in acute infection compared to CHB patients.[2,3]

The immune repertoire includes T cell receptor (TCR) repertoire and B cell receptor (BCR) repertoire. The diversity of TCR/BCR is generated through combinatorial and junctional diversity, and in turn, the specificity of T cell and B cell is determined by its unique TCR and BCR. Earlier researchers revealed that NAs treatment in CHB patients partially restores immune function and leads to partial recovery of HBV-specific T cells.[4] Further longitudinal study on the dynamic changes of CD4 and CD8 TCR repertoires indicated a vigorous T cell expansion in CHB patients with HBeAg seroconversion upon NAs treatment.[5,6] Compared to week 12, more potential HBV-specific T cells emerged at week 48.[7] We also analyzed the immune repertoire features of tumor and adjacent non-tumor tissues from hepatocellular carcinoma (HCC) patients. BCR exhibited higher richness and evenness in non-tumor tissues, while TCR diversity was comparable in tumor and non-tumor tissues.[8] A significant clonal expansion in CD8 but not in CD4 TCR repertoire was observed in HBV-related acute-on-chronic liver failure patients during disease progression.[9] Other study found significant clonal expansion by analyzing BCR repertoire in HBV-related acute-on-chronic liver failure patients.[10]

However, it remains unclear whether there are differences in the impact of NAs treatment on immune repertoires between CHB and HBV-related HCC patients, considering the differences in duration of HBV infection and viral levels between these two groups. Understanding the effect of NAs treatment on the immune repertoire of both CHB and HCC patients is crucial for devising more rational immunotherapeutic strategies. In this study, we analyzed the immune repertoire in the peripheral blood of CHB and HCC patients to clarify the effects and differences of NAs therapy on their T and B cell compositions.

Materials and Methods

Patient Samples

We enrolled CHB patients and HBV-related HCC patients from Nanfang Hospital (Guangzhou, China) and Guangzhou Eighth People's Hospital. The inclusion criteria for CHB patients are as follows: (1) HBsAg or HBV-DNA positive for a duration of 6 months or longer; (2) alanine aminotransferase (ALT) repeatedly or continuously abnormal (2 times or more the upper limit of normal), or histological evidence indicating the liver is in a state of chronic inflammation. The inclusion criteria for HCC patients are as follows: (1) HBsAg and HBV DNA positive for at least 6 months; (2) the imaging evidences of HCC in high-resolution CT or MR imaging, or histopathological diagnosis of HCC. The common exclusion criteria for both CHB and HCC patients are as follows: (1) patients who have previously received or are currently receiving interferon antiviral therapy; (2) patients with co-infections of HAV, HCV, HDV, or HEV; (3) patients infected with HIV; (4) patients with any autoimmune diseases or those taking immunosuppressants; (5) patients with alcoholic liver disease or other metabolic liver diseases. Separately, the exclusion criteria for CHB patients are those with any malignancies. While the exclusion criteria for HCC are as follows: (1) patients with any primary malignancies other than hepatocellular carcinoma; (2) patients who have received or are currently receiving antitumor therapy.

All patients were divided into treated group and untreated group. Patients in the treated group received at least 12 weeks of NAs treatment, while patients who had never received any approved antiviral treatment belonged to the untreated group (2 HCC patients treated with NAs for 2 days were enrolled). In addition, to compare the influence of HBV infection on the immune repertoire, 361 healthy individuals without HBV or HCV infection from our previous study were included as healthy controls.[11] More males than females were involved in CHB and HCC patients. In addition, previous studies have illustrated the differences of adaptive immunity between male and female in healthy adults.[11,12] Therefore, we conducted separate analyses for male and female patients.

Sample Collection and RNA Preparation

Peripheral blood samples were collected with dipotassium ethylene diamine tetraacetate (EDTA-K2) tubes. Then, the total RNA was extracted from 250μL peripheral blood using TRIzol LS Reagent (Invitrogen, USA) according to the manufacturer's instructions.

Library Preparation and Repertoire Sequencing

Unbiased T cell receptor β chain (TCRβ) and B cell receptor immunoglobulin (Ig) heavy chains (BCRh) libraries were prepared using 5′ rapid amplification of cDNA ends strategy, as previously described.[11,13] Briefly, cDNA synthesis reaction was performed using 3μL of total RNA, SMARTer IIA oligonucleotide, SMARTScribe reverse transcriptase (SMARTer PCR cDNA synthesis kit, Clontech, USA), and 4 primers specific to TCRβ, IgA, IgG, IgM chains, respectively. Subsequently, two-round PCR was conducted by using Advantage 2 polymerase mix (Clontech, USA) with IgA, IgG, IgM and TCRβ specific reverse primers and universal forward primers. TCRβ and IgH chains were amplified separately in the second round of PCR. Then, the PCR products were gel-extracted and purified using Qiaquick Gel Extraction kit (Qiagen, Germany). After Illumina adaptors and barcodes were ligated to the libraries, sequencing was performed on the Illumina HiSeq platform with a read length of 2×150bp. The FASTQ files obtained by sequencing were imported to MiXCR (version 3.0.7) for TCR and BCR V, D and J gene identification, CDR3 sequence quantitation and PCR error correction.

We evaluated the diversity of the TCRβ and BCR IgH repertoire from two perspectives: richness and evenness. The richness is represented by the number of unique CDR3nt clonotypes. The evenness, which was estimated by normalized Shannon diversity entropy (NSDE), describes the degree of clonal expansion in the repertoire. The NSDE is calculated as:

$$NSDE = -\sum_{i=1}^{N} \frac{p_i \ln p_i}{\ln N}$$

where p_i is the frequency of the ith clonotype; and N is the total number of clonotypes (richness).[11]

Statistical Analysis

All statistical tests and visual graphs were performed using the R V.4.0.3 (https://www.r-project.org/) or using GraphPad prism V.8.0.2 (GraphPad Software, San Diego, CA). For detailed, in the box plots, the Wilcoxon rank sum test was used in the comparisons with only two groups. For comparisons involving more than two groups, by using the "dunnTest" function in R package "FSA", the Kruskal–Wallis test followed by the Dunn post-hoc test was performed, and the final p values were adjusted by the Benjamini–Hochberg method. The Pearson correlation (two-tailed) and linear regression were calculated.

Results

Clinical Data of Patients and Healthy Donors

A total of 211 patients with HBV infection, including 126 CHB patients and 85 HBV-related HCC patients, were enrolled in this research (Table 1, Figure S1). In addition, more untreated patients were observed in HCC (76.47%) than in CHB (32.54%) patients (p=3.85E-10, Pearson's Chi-square).

Profiles of TCRβ and BCRh Repertoires

After sequencing and filtering, a total of 479,910,853 and 517,466,915 clean reads were obtained, respectively, from the total T cells and B cells for subsequent analysis. Among them, a total of 4,288,560 and 5,865,263 unique CDR3 nucleotide (nt) clonotypes for TCRβ and BCRh were found, respectively, in all 211 samples. More details of the sequencing data of each sample could be found in Supplementary Table S1.

Lower Diversity of TCRβ and BCRh in CHB Patients Than in Healthy Participants and HCC Patients

Referred to the previous method, NSDE were calculated to assess the evenness of the CDR3nt sequences, while the unique CDR3nt clonotypes were used to assess the richness.[11] All patients with HBV infection, including CHB and HCC

Table 1 The clinical information of patients involved

	CHB (n=126)	HCC (n=85)	Healthy (n=361)
Gender (male/female)	91/35	75/10	172/189
Age (years): median (range)	43 (17-77)	51 (23-75)	45 (19-83)
Treatment (treated/untreated)	85/41	20/65	0/361
ALT (ULN): median (range)	0.71 (0.20-42.70)	0.98 (0.13-25.10)	n.t.
HBV DNA <1000IU/ml (%)	67.46%	38.82%	n.t.
HBsAg (-/+/n.t.)	0/126/0	0/75/10	361/0/0
HBsAb (-/+/n.t.)	122/3/1	56/10/19	n.t.
HBeAg (-/+/n.t.)	78/48/0	54/19/12	n.t.
HBeAb (-/+/n.t.)	63/63/0	35/37/13	n.t.

Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; n.t., not tested; ULN, upper limit of normal.

patients, were first compared with healthy controls. We found that the HBV infection patients showed a lower richness and evenness of TCRβ (Figure 1A and B) and BCRh (Figure 1C and D) than healthy individuals, both in males and females.

Further comparing CHB, HCC, and the healthy participants, we found that CHB patients were the lowest both in evenness and richness of the TCRβ repertoire. Although there was no significant difference between CHB and HCC in females, CHB still showed a lower diversity trend compared to HCC (Figure 1E and F). In BCRh repertoire, both evenness and richness in CHB were lower than those of the healthy control group, while no significant difference was found in evenness between CHB and HCC (Figure 1G and H). This suggests that there may be clonal expansion of certain T cell and B cell clones in CHB patients. However, this tendency is not evident in HCC.

Lower Diversity of TCRβ and BCRh in Treated Than in Untreated CHB Patients

The previous multiple studies have shown the impact of oral nucleoside drugs on adaptive immunity in CHB patients.[4–7] For further analysis, patients with CHB and HCC were grouped based on whether they had undergone NAs treatment. We found that, in both male and female patients, the TCRβ richness and evenness of patients who underwent NAs treatment were significantly lower than patients who did not receive antiviral treatment or healthy individuals (Figure 2A and B). Further analysis revealed that this difference was only observed between NAs treated and untreated CHB patients, but not in HCC patients (Figure 2C and D). The results in BCRh repertoire were consistent with those of TCRβ (Figure 2E–H), indicating the expansion of certain T/B cell clones in NAs treated CHB patients, leading to a decrease in the richness and evenness of TCRβ/BCRh repertoire. These results suggested that the NAs treatment definitely influences the diversity of TCRβ and BCRh in CHB patients but not in HCC patients.

Correlation of Immune Repertoire Diversity with ALT and HBV DNA Levels in the Patients

Finally, we analyzed the correlation between the diversity of TCRβ and BCRh repertoires with ALT or HBV DNA levels. Regardless of whether in the TCRβ or BCRh repertoire, we found that there was a significant positive correlation between the diversity of TCRβ and ALT or HBV DNA levels in CHB patients (Figure 3A and B and Figure S2A, S2B). A similar trend can be observed in BCRh repertoire (Figure S2C, S2D). However, no significant correlation was found in HCC patients (data not shown).

Since the richness and evenness of TCRβ and BCR IgH in treated CHB patients were significantly lower compared to untreated patients, we further analyzed the correlation based on treatment. No significant correlation was found in TCRβ richness and evenness with liver inflammation level (Figure 3C and D) or with HBV DNA level (Figure S2E, S2F). The correlations in BCRh repertoire between the diversity and the clinical indicators were similar to the correlations in TCRβ

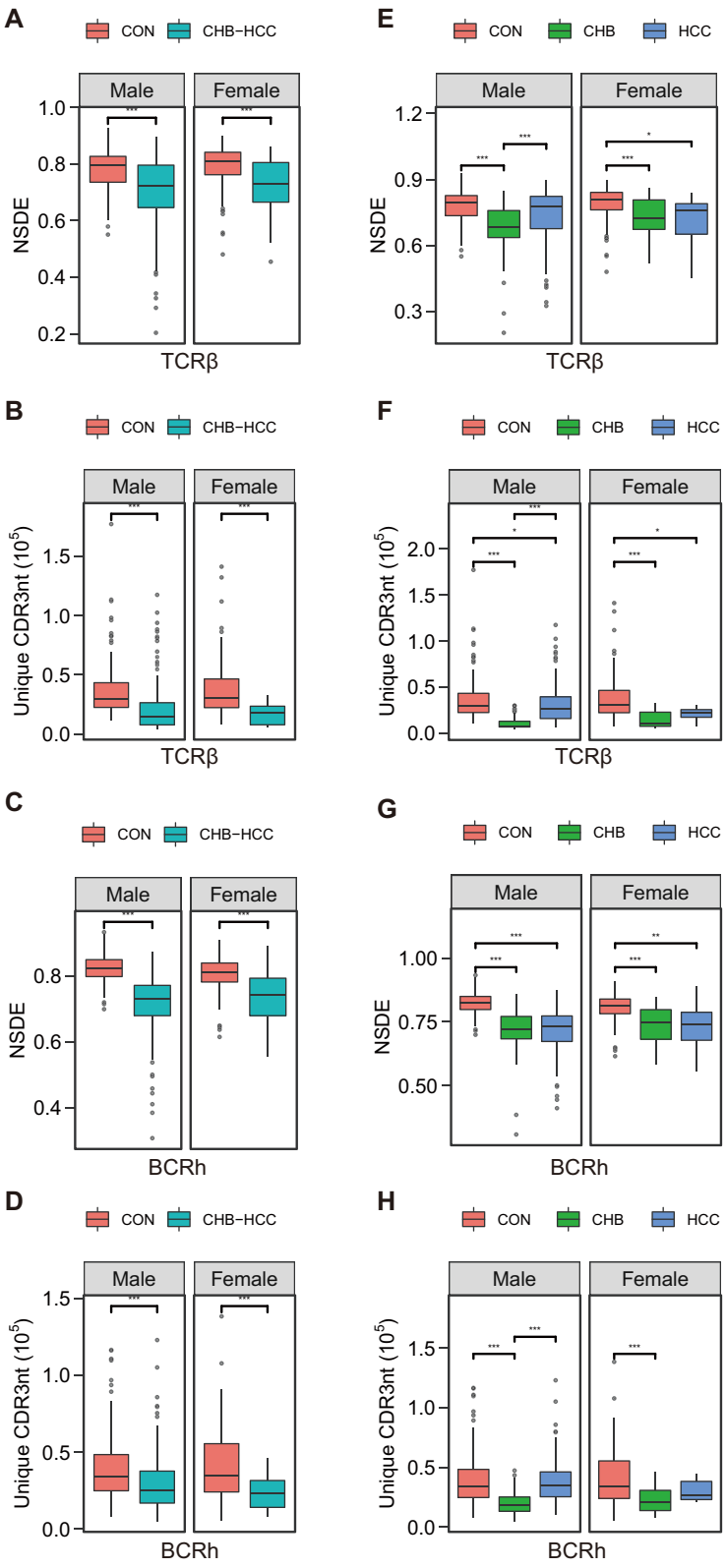

Figure 1 The diversity of the TCRβ and BCR IgH in CHB, HCC and healthy controls. The comparison of the NSDE and the unique CDR3nt between HBV infection and healthy controls, respectively in TCRβ (**A** and **B**) and BCR IgH repertoire (**C** and **D**). The comparison of the NSDE and the unique CDR3nt between CHB, HCC and healthy controls, respectively in TCRβ (**E** and **F**) and BCR IgH repertoire (**G** and **H**). *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: BCRh, B cell receptor heavy chain; CHB, chronic hepatitis B; CHB-HCC, all CHB and HCC patients; CON, healthy control; HCC, hepatocellular carcinoma; NSDE, normalized Shannon diversity entropy; TCRβ, T cell receptor β chain.

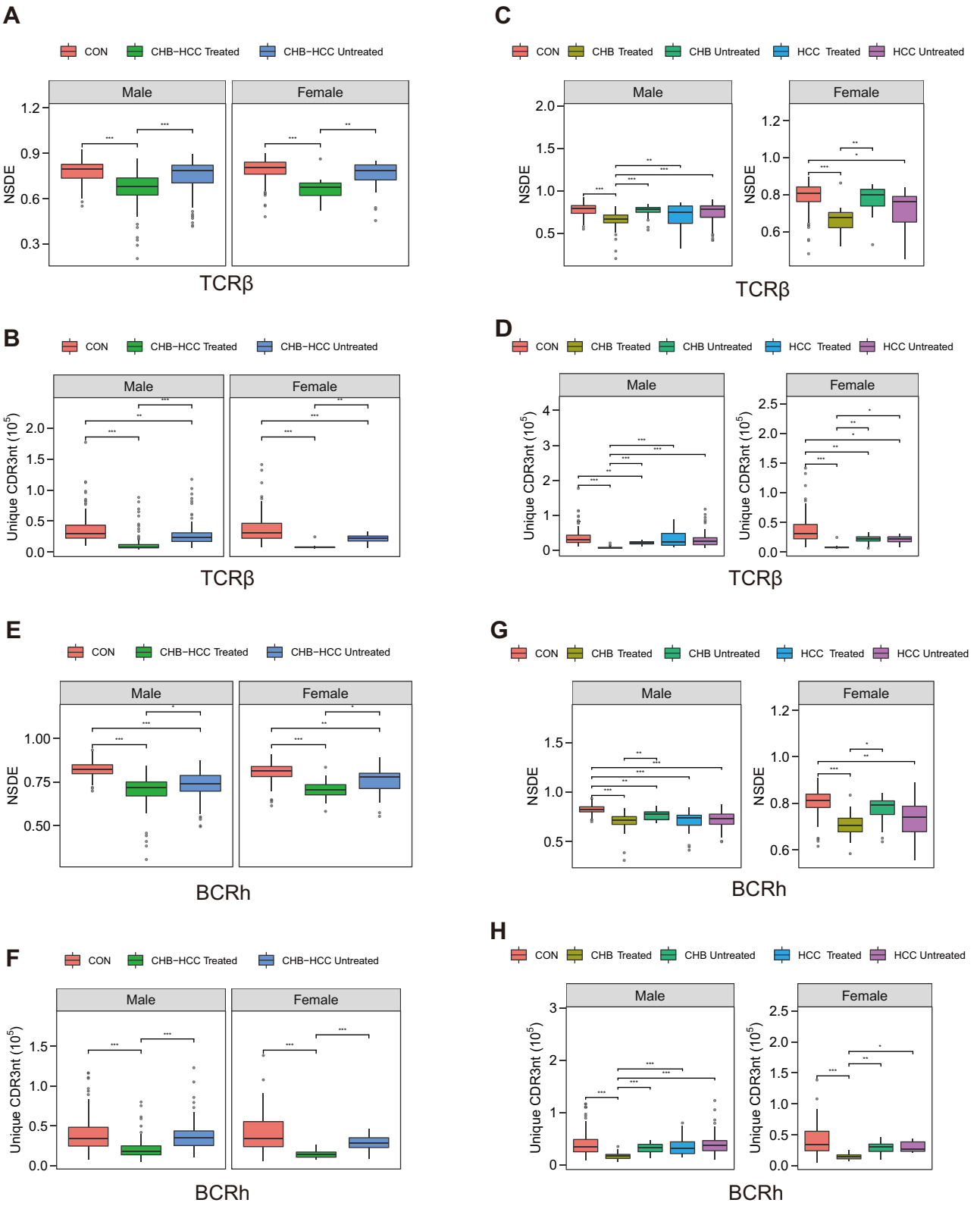

Figure 2 The diversity of the TCRβ and BCR IgH in treated and untreated CHB, HCC. In all HBV infected patients, the NSDE (**A**) and the unique CDR3nt of the TCRβ (**B**) was compared respectively between treated and untreated patients. The NSDE (**C**) and the unique CDR3nt of the TCRβ (**D**) was compared respectively between treated and untreated CHB or HCC patients. The NSDE (**E**) and the unique CDR3nt of the BCR IgH (**F**) was compared respectively between treated and untreated HBV infected patients. The NSDE (**G**) and the unique CDR3nt of the BCR IgH (**H**) was compared respectively between treated and untreated CHB or HCC patients. *p < 0.05, **p < 0.01, ***p < 0.001.
Abbreviations: BCRh, B cell receptor heavy chain; CHB, chronic hepatitis B; CHB-HCC, all CHB and HCC patients; CON, healthy control; HCC, hepatocellular carcinoma; NSDE, normalized Shannon diversity entropy; TCRβ, T cell receptor β chain.

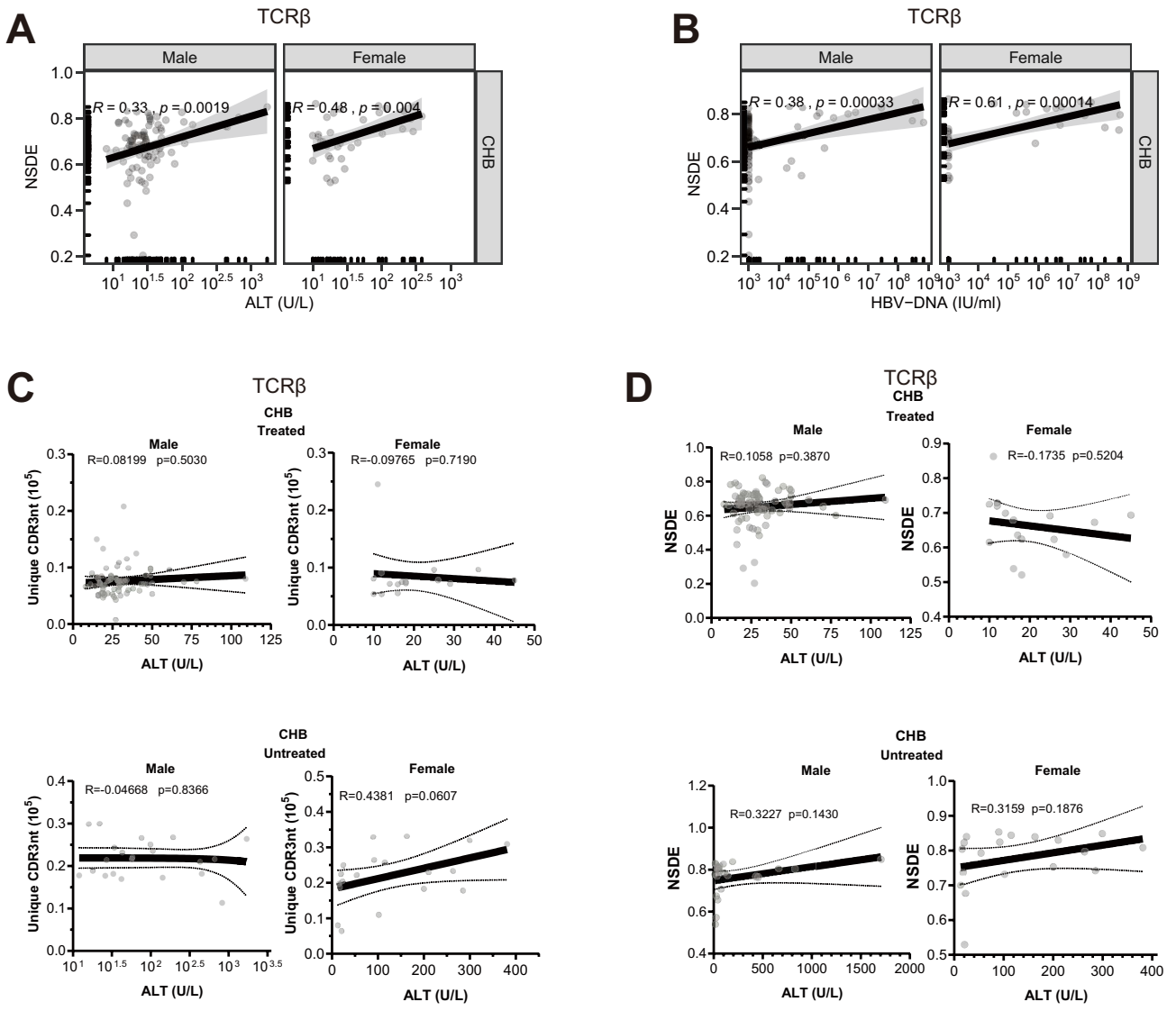

Figure 3 The correlation between the diversity of TCRβ with ALT or HBV DNA levels in CHB patients. (**A**) The correlation between the NSDE of TCRβ and ALT levels respectively in CHB patients. (**B**) The correlation between the NSDE of TCRβ and HBV DNA levels respectively in CHB patients. (**C**) The correlation between the unique CDR3nt of TCRβ and ALT levels in treated CHB patients (above) and untreated CHB patients (below). (**D**) The correlation between the NSDE of TCRβ and ALT levels in treated CHB patients (above) and untreated CHB patients (below).

Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; NSDE, normalized Shannon diversity entropy; TCRβ, T cell receptor β chain.

repertoire (Figure S2G–J). These results suggested that the diversity of TCRβ and BCRh in CHB patients was not directly correlated with ALT or HBV DNA levels.

Discussion

Accumulating evidence demonstrated the heterogeneous of exhausted HBV-specific $CD8^+$ T cells targeting different HBV antigens with distinct phenotypic and functional characteristics in chronic HBV infection patients.[14] The reasons behind the heterogeneity of HBV-specific $CD8^+$ T cells targeting different antigens are still unclear. We have indicated the association of TCR repertoire perturbation with hepatitis B e antigen seroconversion in CHB patients upon NAs therapy.[5,6] The present study demonstrated that NAs treatment significantly affects the clonal composition of T cells and B cells in the peripheral blood of CHB patients but shows less noticeable effects in HCC patients. Our earlier assessment and analysis of potential HBV-specific T cells in these patients indicated that Envelope (S) and Polymerase (P) specific TCRβ clusters tended to be distributed more among younger patients than the Core (C) specific TCRβ clusters. NAs

treatment partially restores the potential HBV-specific T cells in CHB patients.[7] Results from the treatment of CHB and HBV-related HCC patients with nivolumab showed better antiviral immune response in CHB than in HCC.[15,16] These results suggest that initiating antiviral therapy earlier in CHB patients is more beneficial for restoring antiviral immune function. Whereas, for HBV-related HCC patients, due to potentially lower levels of HBV-specific T cells and their more exhausted state, along with a more pronounced immune-suppressive state in the liver, mere antiviral therapy might not significantly impact the body's immunity.

Not surprisingly, no correlation was observed between the diversity of immune repertoire and ALT or HBV DNA levels in CHB patients with NAs therapy. One important reason is that in patients receiving NAs treatment, the majority of patients have maintained normal levels of ALT and HBV DNA levels below the detection limit. Further longitudinal cohort studies are needed to demonstrate the correlation between them. Moreover, it remains to be clarified which antigen-specific T cells are more influenced by NAs treatment or which antigen-specific T cells are more favorably restored. S-specific T cells declined with age and are extremely difficult to detect, whereas T cells specific for C and P persisted.[17] Our recent analysis indicated a more evident recovery of C-specific T cells with NAs treatment.[7] Considering that most HCC patients have had HBV infection for several decades, the relatively minor impact of NAs treatment on T cells in HCC patients might be partly related to this.

Previous studies have analyzed the changes of BCR repertoire in HCC and HBV-related acute-on-chronic liver failure patients, but there is a lack of reports on the impact of NAs therapy on the BCR repertoire of patients with HBV infection. Our previous investigation demonstrated the association of BCR in memory B cells and CD4 TCRβ repertoires upon antibody response to hepatitis B vaccination in healthy adults.[13] In this study, the diversity of BCRh repertoire was generally consistent with the results of TCRβ repertoire, which was expected considering the interdependency between B cells and $CD4^+$ T cells in adaptive immunity. Further research is needed to understand the affected cell subsets and functions. In addition, although age has different effects on the immune repertoire of males and females, no sex differences were observed in the impact of NAs therapy on the immune repertoire of CHB patients in this study. On one hand, it suggests that NAs therapy has a much greater impact on the immune repertoire than age in CHB patients; on the other hand, the proportion of female patients should be increased in the relatively older HCC group to clarify the impact of NAs therapy on their immune repertoires.

Our results showed that the NAs therapy could skew the total T cell diversity in CHB patients. HBV-specific $CD8^+$ T cells play a crucial role in HBV immunological control. Although NAs treatment can partially restore antiviral immune function in CHB patients, this restoration is not sufficient to completely control and clear HBV replication. The majority of patients still require long-term medication. Hence, how to combine immunotherapy with NAs therapy to achieve functional cure on the basis of inhibiting virus replication is currently a focus and challenge. Currently, strategies to restore or increase HBV-specific T cells in CHB patients include immune checkpoint inhibitors, therapeutic vaccines, and adoptive transfer of engineered HBV-specific T cells.[1,18] However, due to the heterogeneity and limited quantity of HBV-specific T cells in CHB patients, the potential of immune checkpoint inhibitors to restore specific T cells is restricted, leading to limited clinical benefits in treating CHB patients. The clinical efficacy of therapeutic vaccines also requires further improvement.[19] The potential of adoptive T cell transfer in curing CHB is still under exploration.[20,21] Considering the relatively stable frequency of C-specific T cells and their more common memory-like phenotype, along with the rarely detectable S-specific T cells and the higher exhaustion status of P-specific T cells, further studies are required to clarify which antigen-specific T cells are more suitable for targeting in the future immunotherapy of CHB patients.

In addition, our results here also showed that, unlike in CHB patients, the impact of NAs therapy on the adaptive immunity of the HCC patients is minimal. Recently, the adoptive transfer of HBs-specific TCR redirected T cells into advanced HBV-related HCC patients has shown certain anti-tumor activity and antiviral effects shown by declined serum HBsAg levels.[21] More recent study in HBV-related HCC patients indicated tumor-infiltrating HBV-specific CD8+ T cells targeting C and P exhibited longer-term relapse-free survival.[22] While integrated HBV fragments in tumor cells mainly express surface antigen, $CD8^+$ T cells specific to C and P also showed active involvement and effective anti-tumor response. Hence, the mechanisms behind the anti-HCC effects of HBV-specific T cells targeting C and P antigens deserve further exploration. Given that NAs therapy has a relatively small impact on the immune repertoire of HCC patients, other strategies that can activate the intrahepatic immune response should be considered for anti-tumor treatment of HCC patients in addition to adoptive T cell therapy.

The levels of HBV DNA and antigens in CHB patients are usually higher than in HCC patients. Therefore, NAs treatment has a greater impact on these HBV-related indicators in CHB patients, subsequently affecting HBV-specific TCR repertoires, including $CD4^+$ and $CD8^+$ T cells. In our study, we observed significant changes in immune repertoires of CHB patients undergoing NAs treatment. However, not all of these significantly altered T cells are likely to be HBV-specific. Therefore, beyond HBV-specific T cells, further research is needed to understand whether NAs treatment affects the frequency of other T cells, such as bystander T cells, as indicated by recent study.[23] HBV-specific T cells not only express immune checkpoint molecules like PD-1 but also exhibit heterogeneous phenotypic molecule expression. The differentiation, development, and functionality of different subgroups of these cells also warrant further investigation.

There are some limitations in the study. First, we only analyzed the effect of NA therapy on the diversity of TCR/BCR repertoire and did not validate the HBV specificity of clones with significant expansion. Due to the low frequency of HBV specific T/B cells in the peripheral blood of CHB patients and the need for precise pairing of TCR/BCR double chains to verify their antigen specificity, there is a need to develop more sensitive and convenient methods for detecting specific T/B cells. Secondly, HBV-related HCC patients are mainly male patients, and our inclusion of female HCC patients is relatively small. Although we observed the similar effects of NAs therapy on the immune repertoire changes between males and females in CHB patients, it is still unclear whether NAs therapy has an impact on the immune repertoire of female HCC patients. Thirdly, it remains to be clarified which cell type is mainly affected by NAs therapy in the immune repertoire, such as $CD4^+$ or $CD8^+$ T cells.

Conclusion

In conclusion, through the analysis of immune repertoire between CHB and HCC patients treated with NAs, we demonstrated that NAs treatment had a significant impact on the diversity of TCR and BCR repertoires in CHB patients, but had no significant effect on HCC patients. This suggests that CHB patients are more likely to achieve the reconstitution of antiviral immune response by NAs treatment compared to HCC patients.

Abbreviations

ALT, alanine aminotransferase; BCRh, B cell receptor heavy chains; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFN-α, interferon-α; NAs, Nucleos(t)ide analogues; TCRβ, T cell receptor β chain.

Data Sharing Statement

The sequencing raw data of 361 healthy individuals have been deposited in GSA (Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences) and the accession number is HRA000887. The sequencing raw data of CHB and HCC patients are available from the corresponding author on reasonable request.

Ethical Statement

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Nanfang Hospital and Guangzhou Eighth People's Hospital. All patients provided written informed consent.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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