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ORIGINAL RESEARCH

RNA Sequencing Analysis of Patients with Chronic Hepatitis B Treated Using PEGylated Interferon

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Purpose: Worldwide, chronic hepatitis B virus (CHB) infection is a public health concern, ultimately leading to liver cirrhosis and hepatocellular carcinoma. Currently, patients with CHB can be treated using polyethylene glycol (PEG)ylated interferon (PEG-IFN) antiviral therapy, which has both immune modulatory and antiviral properties. This study aimed to reveal the mechanism underlying the effect of PEG-IFN therapy, to rationally optimize this therapeutic option.

Patients and Methods: Ten patients with CHB who were positive for the hepatitis B virus e antigen (HBeAg) and were receiving PEG-IFN treatment were enrolled. Clinical and virological parameters were monitored during 48 weeks of treatment. In addition, peripheral blood mononuclear cells (PBMCs) were collected from the 10 patients at 0, 24, and 36 weeks. RNA sequencing technology was used to analyze the RNA expression profile in the PBMC samples.

Results: Following PEG-IFN treatment, we identified 217 differentially expressed genes (DEGs), most of which were upregulated. Gene ontology enrichment analysis of the DEGs revealed that they were enriched in 29 clusters, mainly associated with "antiviral defense", "innate immunity", "immunity", "defense response to virus", "response to virus", "type I interferon signaling pathway", "negative regulation of viral genome replication", "innate immune response", and "RNA-binding".

Conclusion: After PEG-IFN treatment, a certain mRNA expression profile was observed in patients with CHB, providing further mechanistic insights into the antiviral effect of this therapy.

Keywords: Chronic infection, Hepatitis B, PEGylated interferon, RNA sequencing

Introduction

Hepatitis B virus (HBV) comprises a small noncytopathic DNA virus that can infect hepatocytes and cause diverse outcomes, such as acute hepatitis B and chronic infection. The prevalence of HBV infection is defined by the positivity of serological testing of hepatitis B surface antigen (HBsAg). Areas where HBV is endemic include Asia, the Pacific islands, Africa, Southern Europe, and Latin America.[1,2] Chronic hepatitis B (CHB) virus infection impacts 296 million people around the world, being a major causative agent of hepatocellular carcinoma and cirrhosis, which lead to more than 820000 deaths annually.[3,4] Currently, the two main antiviral therapies for patients with CHB are nucleos(t)ide analogues (NA) and polyethylene glycol (PEG)ylated interferon alfa (PEG-IFNα).[5] The former includes tenofovir alafenamide (TAF), tenofovir (TDF), telbivudine (LdT), entecavir (ETV), adefovir (ADV), and lamivudine (LAM), which can inhibit viral replication and reduce the viral load. Among them, ETV, TDF, and TAF are the first-line drugs of choice because of their high antiviral potency and low resistance rates.[6] In comparison to NAs, PEG-IFNα, including PEG-IFNα-2a and α-2b, has both immune modulatory and antiviral properties.[7] For patients with CHB, PEG-IFN therapy has the benefits of a finite treatment course, a long-lasting therapeutic response, higher rates of hepatitis B e antigen (HBeAg) seroconversion, and no risk of drug resistance. However, PEG-IFN has some disadvantages, such as a moderate antiviral effect, only a subset of patients showing a sustained response, subcutaneous injection, high rates of adverse

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events, and high cost.[8] Furthermore, investigations into the mechanism of the effects of IFNα have revealed a clear role for IFNα treatment in antiviral activity and an immunoregulatory effect.[9] IFNα is a crucial modulator of both adaptive and innate immune responses, shaping the landscape of the immune system to coordinate various immune cells.[10] In our previous study, a certain pattern in the levels of cytokines was observed during IFNα therapy. Moreover, the early expression pattern of cytokines might be a biomarker for treatment response.[11] However, the exact mechanism remains unknown.

Historically, quantitative real-time reverse transcription PCR (qRT-PCR) is used for single gene tests and is generally deemed as the "gold standard" measurement. However, it has a major limitation that only a few measurements can be made in a single assay.[12] With the evolution of deep sequencing, our understanding of biology and medicine has greatly expanded. RNA sequencing (RNA-Seq) was developed to investigate diverse RNA species, and has provided new opportunities for the study of a variety of diseases, such as infectious diseases and cancers.[12] Currently, the use of RNA-Seq in infectious diseases includes RNA-based pathogen diagnostics, measurement of microbial exogenous small RNAs, pathogen mRNAs and host RNAs. Although studies have reported the application of RNA-Seq in HBV,[13,14] its involvement in exploring the mechanism of PEG-IFN treatment in patients with CHB is limited. Thus, herein, we used RNA-Seq to investigate the early expression pattern of mRNAs in patients with CHB receiving PEG-IFN treatment.

Materials and Methods

Patients and Sample Collection

This study enrolled 10 patients with HBeAg-positive CHB who were attending the Department of Infectious Disease, Huashan Hospital, Fudan University, China. The inclusion criterion was patients confirmed to have been HBsAg positive for more than 6 months. The exclusion criteria included: coexisting serious medical or psychiatric illness, cytopenia, coinfection with hepatitis C or D virus or the human immunodeficiency virus, and decompensated liver disease. These 10 patients were treated with PEG-IFNα for 48 weeks between 2013 and 2015. We monitored clinical and virological parameters during the 48 weeks of treatment, including HBsAg, HBeAg, HBV DNA, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Peripheral blood mononuclear cells (PBMCs) were collected from the 10 patients at 0, 24, and 36 weeks and stored at –80 °C. The study was performed following to the ethical standards for human experimentation of Huashan Hospital, Fudan University, and according to the tenets of the Declaration of Helsinki. Each participant provided written informed consent.

Preparation of RNA and Sequencing

Trizol was employed to extract total RNA from 20 samples of PBMCs from the 10 patients following the supplier's recommendations (Invitrogen, Carlsbad, CA, USA). Then, a TruSeq™ RNA sample preparation Kit (Illumina Inc., San Diego, CA, USA) was used to generate the RNA-seq transcriptome libraries from 5 μg of total RNA. In brief, oligo (dT) beads were used to isolate polyA-mRNA, which was then fragmented employing fragmentation buffer (Truseq™ RNA sample preparation Kit). Next, cDNA was synthesized, end repaired, added with an A-base, and ligated to NGS-indexed adaptors (Truseq™ RNA sample preparation Kit). Then, the libraries were subjected to size selection for the appropriately-sized cDNA fragments followed by 15 cycles of PCR amplification utilizing Phusion DNA polymerase (NEB, Ipswich, MA, USA). The amplicons were then quantified using a fluorometer (TBS380, Promega, Madison, WI, USA). Subsequently, a NovaSeq 6000 system (2 × 150 bp, Shanghai BIOZERON Co., Ltd, Shanghai, China) was used to sequence the paired-end libraries.

Analysis of Differentially Expressed Genes (DEGs) and Their Functional Enrichment

To determine the DEGs between the baseline and on-treatment samples, each gene's expression level was calculated according to the fragments per kilobase of exon per million mapped reads (FPKM) method, as executed using the cufflinks software (http://cole-trapnell-lab.github.io/cufflinks/). RSeQC-2.3.2 software was then used to analyze the gene coverage (http://code.google.com/p/rseqc/). SAM (significance analysis of microarrays; Raybiotech, Norcross, GA, USA) was performed to find the DEGs after PEG-IFN treatment. The DAVID Bioinformatics Database was used to

perform Gene Ontology (GO) functional enrichment of the DEGs (http://david.ncifcrf.gov/home.jsp). DEGs with a p-value < 0.05 were deemed to be enriched significantly for a GO term.

Statistical Analysis

Statistical analyses were performed using SPSS software (IBM Corp, Armonk, NY, USA). Skewed laboratory values were log transformed prior to analysis. Data are shown as the mean ± SD or as a frequency (%). Associations between variables were tested using Chi-squared and Student's *t*-tests (or their appropriate non-parametric equivalents). A probability (p) value < 0.05 was employed to represent statistical significance.

Results

Characteristics of the Patients

We enrolled 10 patients with HBeAg-positive CHB receiving PEG-IFNα treatment into the study. Their baseline clinical characteristics are summarized in Table 1. These patients received PEG-IFNα monotherapy or PEG-IFN and NAs combination therapy for 48 weeks. After 48 weeks of treatment, a virological response (as defined as loss of HBeAg and HBV DNA) was achieved by 4 patients, but not by the other 6. The baseline characteristics of the responders and non-responders were similar (P > 0.05). Figures 1 and 2 show the dynamic levels of AST, ALT, HBV DNA, HBsAg, and HBeAg in the virological responders and non-responders during treatment.

Identification of DEGs Associated with PEG-IFN Therapy

We performed RNA-seq on 20 PBMC samples from the 10 patients with HBeAg-positive CHB treated with PEG-IFNα. Gene coverage analysis showed no bias and the samples were uniform. We performed quantile normalization for every sequence, which was then used for screening. The screening criteria were as follows: At least 20% (four examples) had mean changes greater than 1.1× and no missing values. After screening, a total of 12741 genes were detected for subsequent analysis. The SAM method was then carried out to find DEGs after PEG-IFN therapy, which identified a total of 217 DEGs (Figure 3). The results of heat map analysis indicated that most of the 217 DEGs were upregulated after the IFNα treatment.

GO Enrichment Analysis of DEGs

These 217 DEGs were subjected to GO enrichment analysis in the DAVID Bioinformatics Database to reveal their functional classification. The results demonstrated that they were enriched in 29 clusters. We further analyzed the cluster with highest enrichment score (15.7) and detected that this cluster comprised nine GO categories including "antiviral defense", "innate immunity", "immunity", "defense response to virus", "response to virus", "type I interferon signaling pathway", "negative regulation of viral genome replication", "innate immune response" and "RNA-binding" (Figure 4). These findings suggested that the potential mechanism of PEG-IFN therapy was related with its direct antiviral defense and immunoregulatory effects, especially innate immunity.

Table 1 Baseline Clinical Characteristics of the 10 HBeAg-Positive Patients with CHB Receiving PEG-IFN-α Treatment

	Response (N = 4)	No response (N = 6)	P
Male (%)	75 (%)	83.33 (%)	
Age (years)	29.67 ± 5.51	29 ± 4.34	0.847
HBV DNA (log IU/mL)	7.20 ± 7.28	7.22 ± 7.19	0.943
HBsAg (log IU/mL)	3.69 ± 3.73	4.34 ± 3.38	0.160
HBeAg (S/CO)	286.15 ± 233.33	502.70 ± 446.37	0.347
ALT (U/L)	244.75 ± 217.40	126.83 ± 92.49	0.263
AST (U/L)	105.25 ± 56.16	58.17 ± 31.97	0.126

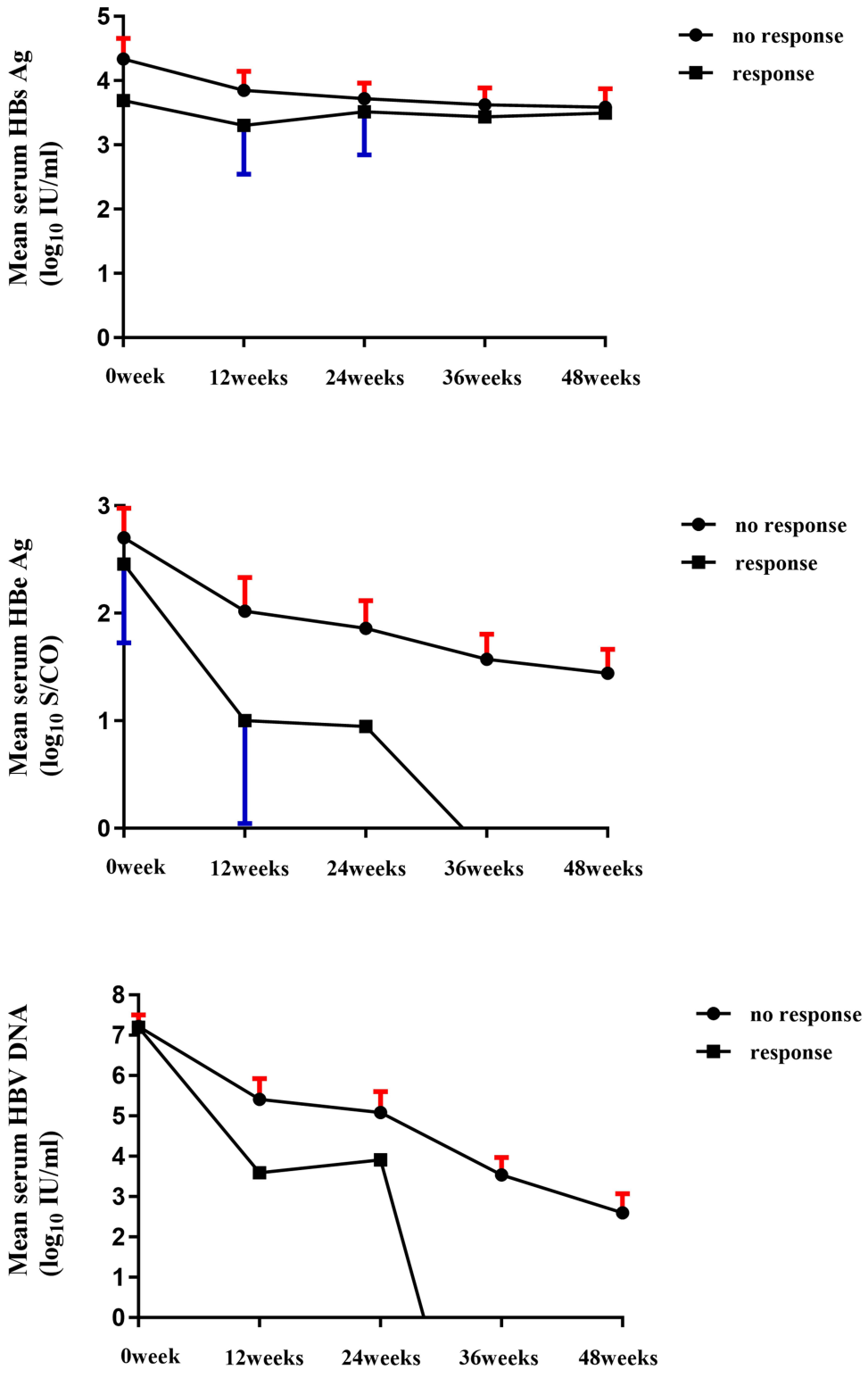

Figure 1 Hepatitis B virus (HBV) DNA, hepatitis B e antigen (HBeAg), and hepatitis B surface antigen (HBsAg) levels in virological responders and non-responders among the 10 HBeAg-positive patients with chronic hepatitis B (CHB) during PEG-IFN therapy for 48 weeks.

Identification of Potential Predictive Genes for PEG-IFN Therapy

Subsequently, we selected three categories from the cluster of highest enrichment scores for further analysis: the "type I interferon signaling pathway", "innate immunity", and "antiviral defense". As shown in Figure 5, increased expression

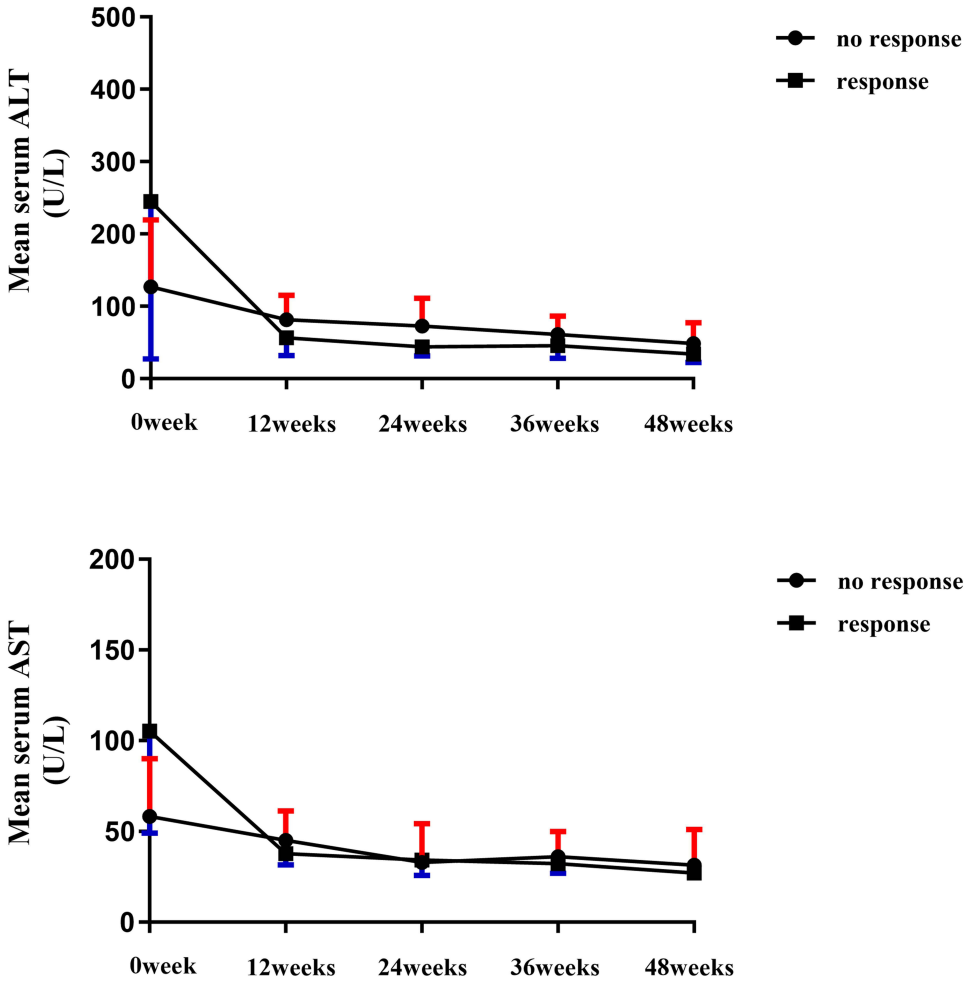

Figure 2 The dynamic amounts of aminotransferase (ALT) and aspartate aminotransferase (AST) in virological responders and non-responders among the 10 HBeAg-positive patients with chronic hepatitis B (CHB) during PEG-IFN therapy for 48 weeks.

levels of most genes from these three categories were observed after PEG-IFN treatment. To further explore the potential DEGs that could predict the treatment response, the fold change in gene expression between the on-treatment level and the baseline level was used as a parameter. Thirty-nine genes from the three categories were enrolled for the analysis. However, the results showed no significant differences in the fold changes between the virological responders and non-responders for the 39 genes ($P > 0.05$).

Discussion

The World Health Organization (WHO) has called for the eradication of HBV by 2030, defined as a 90% reduction in new infections and a 65% decrease in deaths in comparison with the baseline levels in 2015.[3] A major challenge to meet this target is covalently closed circular DNA (cccDNA) in hepatocyte nuclei, which acts as a transcriptional template for all HBV RNAs, causing difficulty in completing the elimination of HBV.[15] Currently available therapy regimens to treat patients with CHB remain as NAs and PEG-IFNα. Therapy with NAs lacks a direct inhibitory effect on cccDNA, resulting a minimal reduction in the cccDNA concentration, even after many years of treatment. By contrast, IFNα treatment has direct inhibitory effects on cccDNA, suppressing cccDNA transcription and inducing its degradation.[7] These advantages make PEG-IFN therapy a first-line treatment for patients with CHB pursuing a functional cure. Moreover, further exploration of the underlying mechanism of the effects of PEG-IFN treatment will help patients to gain further benefit from this therapeutic option. Next-generation sequencing (NGS) allows high-throughput identification of DNA sequences and has rapidly become an essential technology in clinical research applications.[16,17] RNA-Seq

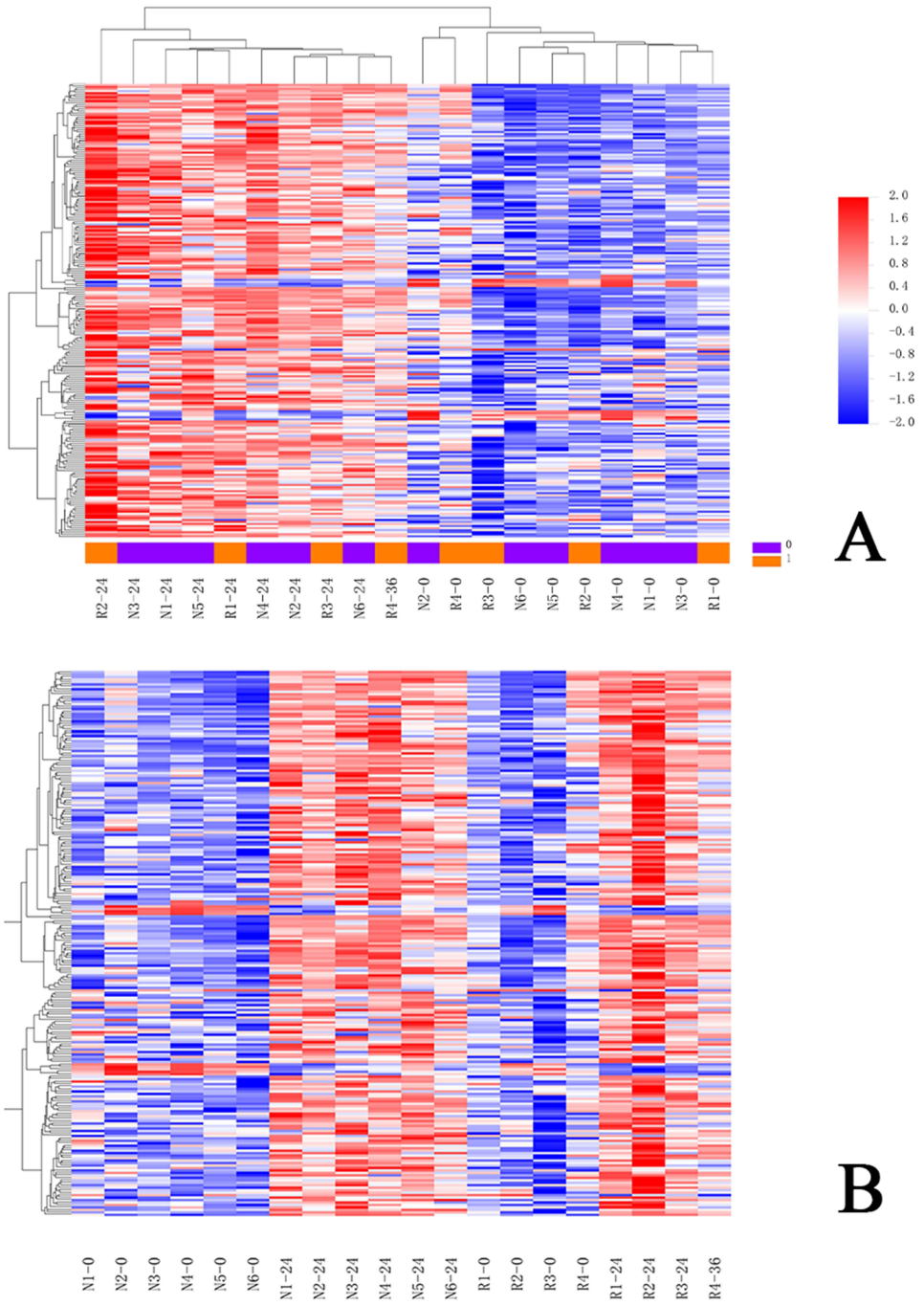

Figure 3 Heat maps of 217 PEG-IFN-related DEGs. R, responder. N, non-responder. For example, R2-24 means 24-week sample of responder number 2. (**A**) Arranged by week. (**B**) Arranged by response or no response.

technology is a cutting-edge NGS approach for surveillance of the whole transcriptome rapidly and quantitatively.[18] The initial applications of RNA-Seq technology were involved in the yeast transcriptome,[19] mouse tissues,[20] human embryonic kidney, and B cell lines.[21] Meanwhile, RNA-Seq has furthered our understanding of the mechanisms underlying liver diseases. Huang and co-workers performed RNA-Seq analysis for HBV-associated hepatocellular carcinoma (HCC), and identified 1378 DEGs and 24338 differentially expressed exons when comparing the cancer and non-cancerous tissues.[22] Functional annotation revealed that the 1378 DEGs were most significantly enriched in 54 bio-function terms and 41 canonical pathways, providing a new mechanistic comprehension of liver carcinogenesis.

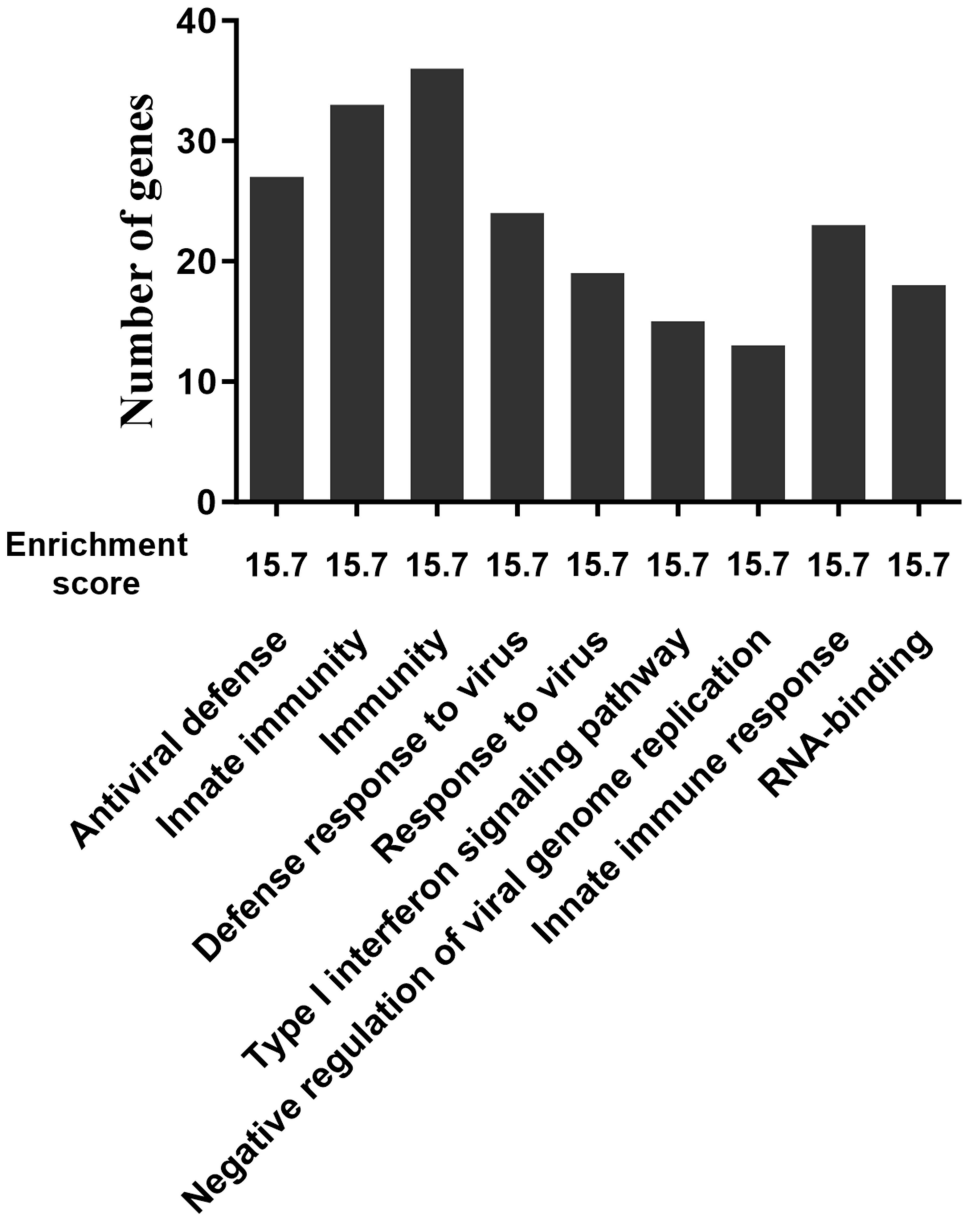

Figure 4 GO enrichment of DEGs with the highest enrichment score of 15.7, including "antiviral defense, "innate immunity", "immunity", "defense response to virus", "response to virus", "type I interferon signaling pathway", "negative regulation of viral genome replication", "innate immune response" and "RNA-binding". The category name is shown on the x-axis. The number of genes in each category is shown on the y-axis.

Herein, 10 patients with CHB were enrolled, who received PEG-IFN-α treatment for 48 weeks. Among these 10 patients, 4 showed a virological response and 6 showed no response. To explore the mechanism of the effect of PEG-IFN in patients with CHB, RNA-Seq was carried out to assess the mRNA expression profile in the 10 patients during PEG-IFN treatment. We detected a total of 217 DEGs, most of which were upregulated following PEG-IFN treatment. GO enrichment analysis of the DEGs indicated that they were mainly enriched in "antiviral defense", "innate immunity", "immunity", "defense response to virus", "response to virus", "type I interferon signaling pathway", "negative regulation of viral genome replication", "innate immune response", and "RNA-binding". Research has demonstrated that PEG-IFN therapy could improve innate immunity through the restoration of natural killer cell effector functions.[23,24] This was confirmed in the present study, which provided further mechanistic insights into the immunoregulatory impact of PEG-IFN treatment. Moreover, previous studies have identified the relationship between IFN-based therapy and the interferon signaling pathway.[25–27] Activation of the interferon signaling pathway could induce the intrinsic antiviral proliferation

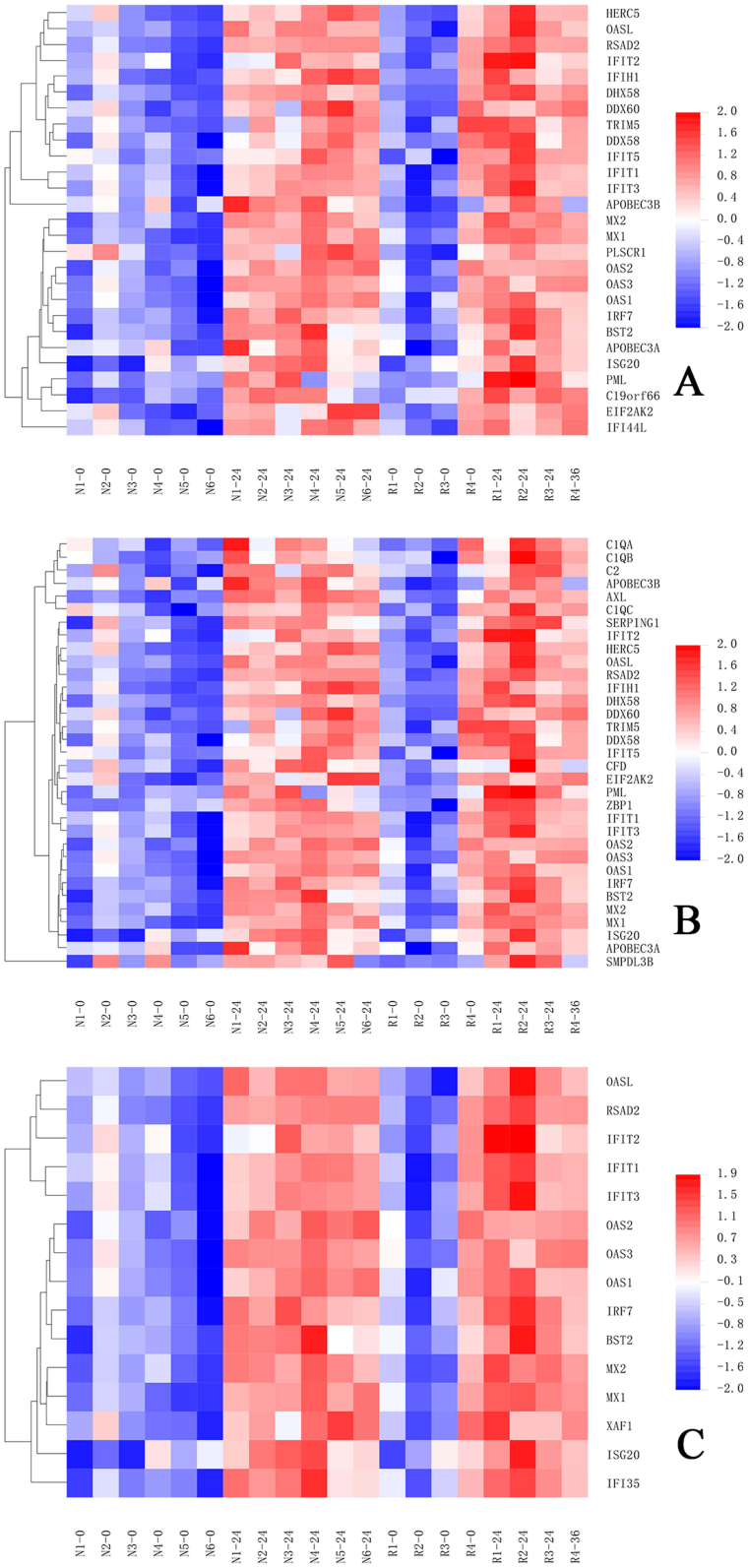

Figure 5 Heat maps of three categories including "type I interferon signaling pathway", "innate immunity", and "antiviral defense". R, responder. N, non-responder. For example, N1-0 means baseline sample of non-responder number 1. (**A**) "Antiviral defense" (**B**) "Innate immunity" (**C**), "Type I interferon signaling pathway".

activity of cells and promote immune cell activation. In our study, the expression levels of genes related to the interferon signaling pathway were unregulated after PEG-IFN treatment, suggesting that activation of interferon signaling and its stimulation of downstream genes might play important roles in the therapeutic effect of PEG-IFN. To explore potential DEGs that could predict treatment response, we further evaluated 39 genes from three categories of "type I interferon signaling pathway", "innate immunity", and "antiviral defense". However, we did not find any genes that could predict the PEG-IFN therapy response. This could be because of the small number of patients that were enrolled in the study; thus, additional experiments with larger cohort of patients are warranted to identify and verify genes that are predictive of treatment response.

Conclusion

We identified 217 PEG-IFN treatment-associated DEGs, which were mostly enriched in "antiviral defense", "innate immunity, immunity", "defense response to virus", "response to virus", "type I interferon signaling pathway", "negative regulation of viral genome replication", "innate immune response", and "RNA-binding". These results allowed us to gain novel mechanistic insights into the therapeutic effect of PEG-IFN treatment. However, our study enrolled only few patients; therefore, a large cohort study is required to further explore the mechanism and confirm predictive genes for the effect of PEG-IFN therapy.

Ethical Approval

This study received approval from the Ethics committee of Huashan Hospital, Fudan University. All participants provided written informed consent. The study adhered to the principles outlined in the Declaration of Helsinki.

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Disclosure

The authors declare that they have no conflict of interest.

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