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CDR3 repertoire diversity of CD8+ T lymphocytes in patients with HCV

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ABSTRACT

T cell receptors (TCR) diversity is known to serve as a defining hallmark of the antigen-reactive T cell repertoire. Complementarity determining region 3 (CDR3) was the most important region for the recognition of peptide-major histocompatibility complex (MHC) complexes and represented the diversity of TCR repertoire. In this study, we detected the CDR3 spectratypes by complexity scoring system to assess TCR repertoire diversity and further analyzed the correlation of CDR3 score with CD8+ T cell function and with the prognosis of chronic hepatitis C virus (HCV)-infected patients. The results demonstrated that CDR3 score was related to CD8+ T cell function and prognosis by analyzing the clinical indicators such as viral load (VL), rapid virologic response (RVR), early virologic response (EVR) and sustained virologic response (SVR). Importantly, we found that Vβ27, a member of CDR3 subfamily, might play an important role in the clearance of HCV. These findings indicate that TCR diversity maybe serve as a biomarker to predict the clinical parameters of HCV-infected patients.

1. Introduction

T lymphocytes are known to protect immune system from attacking by bacteria, virus or cancer cells. T cells recognize the antigens depend on interaction of cell surface T cell receptors (TCR) and peptides presented by the major histocompatibility complex (MHC) [1,2]. In order to enable the recognition of diverse peptide-MHC complexes, the TCR β chain is generated by the chromosomal rearrangement of V, D and J gene segments. In contrast, the TCR α chain undergoes V and J gene segments recombination, resulting in a limited diversity [3]. Moreover, Each V gene segment can be subdivided into three regions of hypervariability (complementarity determining regions (CDRs)). CDR3 is most important for the recognition of MHC complexes among three CDRs [4–6]. Therefore, analyses of CDR3 spectratypes might be useful for defining the degree of clonality of T cells and the diversity of TCR repertoire.

Diversity of TCR repertoire can reach 10^{15} in theory, however, CD8+ TCR repertoire in human adults is in the range of 10^5–10^8 [7–9]. In addition to T cell frequency, TCR diversity is a defining hallmark of the antigen-reactive T cell repertoire. Cytomegalovirus and influenza A virus specific CD8+ T cells repertoires were analyzed and thousands of new TCR were identified [10]. A positive correlation between TCR repertoire normalization and remission of colorectal cancer was observed [11]. *T*-cell repertoire in peripheral blood lymphocytes showed an apparent reversion to a more normal *T*-cell repertoire in Hepatitis-associated aplastic anemia patients after successful treatment [12]. Some unique dominant TCRβ sequences were identified in patients with Refractory coeliac disease type II [13].

Chronic hepatitis C virus (HCV) infection is a major global health problem that represents a major cause of liver cirrhosis and hepatocellular carcinoma (HCC) [14]. In China, the overall prevalence of HCV infection is 2.2%, and there is a prevalence of 9.6% in Henan province [14–16]. HCV is an excellent model for understanding the relationship between pathogens and host cell pathways [17]. In this study, we mainly analyze CDR3 spectratypes of CD8+ T cells in HCV patients. We aim to verify whether a TCR type, a group of TCR types or TCR diversity could be used as a marker to predict the clinical parameters of HCV patients such as prognosis.

2. Materials and methods

2.1. Reagents

The reagents used in this article are as follows: Anti-CD8 microbeads

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(Miltenyibiotec, Germany); MACS buffer (Miltenyibiotec, Germany); TRIzol (Invitrogen Corporation, Carlsbad, CA, USA); Primescript RT reagent kit (TakaRa, Dalian, China); Ex Taq polymerase (TakaRa, Dalian, China); Human IFN-γ ELISA MAX™ Deluxe Set (Biolegend) and Human TNF-α ELISA MAX™ Deluxe Set (Biolegend).

2.2. Ethics statement

The study was approved by the First Affiliated Hospital of Zhengzhou University. The samples were collected after signing written informed consent. The whole consent procedure was in accordance with the standard defined by ethics committee of the First Affiliated Hospital of Zhengzhou University.

2.3. Human samples

Twenty-nine subjects were enrolled in this study. These patients were hospitalized during January 2014 to January 2015 in the First Affiliated Hospital of Zhengzhou University and the healthy age-matched control donors were offered by Henan Red Cross Blood Center. The patient exclusion criteria included co-infection with human immunodeficiency virus (HIV), hepatitis B virus (HBV), or mycobacterium tuberculosis, alcohol intake averaging > 50 g per day, active drug abuse, chronic systemic disease, psychiatric disorders, autoimmune disease, and pregnancy or lactation. The main clinical and pathologic characteristics of the patients were presented in Table 1.

2.4. CD8+ T cells sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from patients with HCV using density gradient centrifugation. 1×10^7 PBMCs were incubated with 20 μl anti-CD8 microbeads (Miltenyibiotec, Germany) and 80 μl MACS buffer (Miltenyibiotec, Germany). Cells were washed using MACS buffer after incubating 15 min in 4 °C. Then cells were loaded into MS column (Miltenyibiotec, Germany) and positively selected according to the manufacturer's instructions.

2.5. ELISA

IFN-γ and TNF-α in patients' serum were detected using Human IFN-γ ELISA MAX^{tm} Deluxe Set (Biolegend) and Human TNF- α ELISA MAX™ Deluxe Set (Biolegend). Briefly, capture antibodies were diluted and sealed the plates overnight in 4 °C. Next day, the plates were washed and added samples and incubated 2 h. Diluted detection antibodies were added into samples. After incubated for 1 h, Avidin-HRP solution was added and incubated for 30 min. Finally, freshly mixed TMB substrated solution was added for 20 min in the dark and stop solution was used to stop color reaction and the absorbance at 450 nm was read.

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted from CD8+ T cells with TRIzol (Invitrogen Corporation, Carlsbad, CA, USA) and quantified by spectrophotometry. According to the manufacturer's instructions, cDNA was prepared by use of the Primescript RT reagent kit (TakaRa, Dalian, China).

2.7. Quantitative real-time polymerase chain reaction (RT-PCR)

PCR amplification of cDNA was performed with the Vβ primers. Amplifications were performed with 0.5 mM forward primer, 0.5 mM reverse primer end-labeled with a fluorescence tag (6-FAM), 0.2 mM dNTP, 0.5 U Ex Taq polymerase (TakaRa, Dalian, China) and 0.5 μl cDNA in a 20 μl vol. Following denaturation at 94 °C for 5 min, the amplification was carried out with 40 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min.

2.8. Analysis of spectratype

The PCR products were mixed with 2 μl formamide and 0.5 μl loading dye. The mixture was denatures at 95 °C for 2 min and 2 μl was loaded on a 6% acrylamide sequencing gel and run for 2 h on a 50 lane Applied Biosystems model 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA). The data were analyzed by 310 GeneScan Software (Applied Biosystems). CDR3 spectratypes complexity scoring system was used to assass TCR repertoire diversity. Each Vβ subfamily was determined by counting the number of peak per family as described previous [11,12]. Briefly, each Vβsubfamily was given a score of 0 to 5. CDR3 score was 5 if Vβ subfamily spectratypes contained > 5 peaks.

Table 1
Clinical characteristics of patients.

Characteristic	Genotype 1b (n = 18)	Genotype 2a (n = 10)	Genotype 1a (n = 1)	Total (n = 29)
Age (yr)				
Median	50.50	57.50	63.00	52.00
Range	22–70	35–63		22–70
Sex, n (%)				
Male	7(38.89)	4(40)		11(37.93)
Female	11(61.11)	6(60)	1(100%)	18(62.07)
Viral load				
Median	1.07E + 05	1.50E + 01	1.50E + 01	2.38E + 03
Range	1.50E + 01–1.78E + 07	1.50E + 01–2.91E + 06		1.50E + 01–1.78E + 07
ALT				
Median	33.50	22.00	30.00	29.00
Range	8–99	6–547		6–547
AST				
Median	32.00	28.50	37.00	29.00
Range	12–89	11–989		11–989
Lymphocyte (%)				
Median	32.05	31.90	42.50	32.90
Range	11.4–88.1	4.5–46.7		4.5–88.1
Lymphocyte (#10^9)				
Median	1.30	1.35	0.93	1.30
Range	0.3–4.3	0.6–2.2		0.3–4.3

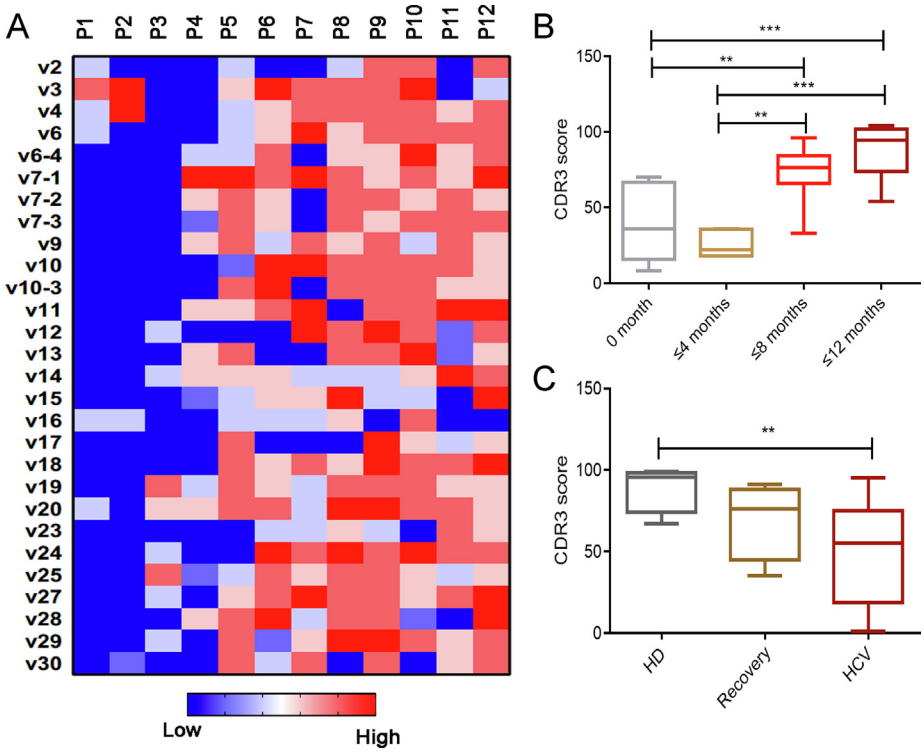

Fig. 1. HCV patients display reduced TCR Vβ CDR3 scores of CD8+ T cells. CD8+ T cells were isolated from PBMC in HCV patients using anti-CD8 monoclonal antibody coupled with magnetic beads. And we detected CDR3 spectratypes of CD8+ T cells. (A) Heat map showed the CDR3 spectratypes of representative patients in different treatment time. P1–P3 didn't receive any therapy; P4–P6 were treated 4 months; P7–P9 were treated between 4 months and 8 months; P10–P12 were treated between 8 months and 12 months. (B) The histogram represented CDR3 spectratypes of 27 HCV patients. (C) CDR3 spectratypes scores were compared among 5 healthy donors, 5 HCV recovery patients and 29 HCV patients. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

The overall spectratype score per sample was calculated as the sum of the scores for each subfamily.

2.9. Statistical analysis

Data were plotted using Graphpad Prism version 6 and Photoshop version Cs5 and $P < 0.05$ was considered statistically significant.

3. Results

3.1. CD8+ T cells CDR3 spectratypes of HCV patients and healthy controls

To determine the difference of spectratypes between HCV patients and healthy control, we enrolled 29 patients infected with HCV, and age-matched health donors. Among these HCV patients, there are 18 genotype 1b, 10 genotype 2a and 1 genotype 1a (Table 1). We used 10 ml blood to sort out CD8+ T cells to detect CDR3 spectratypes and the purity was 95%. Heat map showed the CDR3 spectratypes of representative patients in different treatment time (Fig. 1A). Firstly, CD8+ T cells were sorted from HCV patients and health donors. CDR3 scores were found lower in HCV patients than that in health donors (Fig. 1C). Indeed, patients who had recovered from HCV infection had higher CDR3 scores than unrecovered (Fig. 1C). In contrast to patients without any therapy, HCV patients who received antiviral therapy also showed higher CDR3 scores, and CDR3 scores increased along with the antiretroviral therapy (ART) (Fig. 1B).

3.2. The relationship of CD8+ T cells function and CDR3 scores

CDR3 score was used as a digital standard to evaluate TCR repertoire diversity. Different antigens can be recognized by different TCRs, and T cell function is closely associated with TCR diversity [18]. To define whether CDR3 scores were related to the CD8+ T cell function in HCV patients, we analyzed the function of T cells in patients with high and low CDR3 scores by RT-PCR and ELISA. The HCV patients clinial paratemers of CDR3 high group and CDR3 low group were showed in Table 2. Compared with CDR3 low group, IFN-γ (Fig. 2A) and TNF-α (Fig. 2B) in patients' serum were higher and the RT-PCR results showed that the mRNA expression of granzyme B (Fig. 2C) and perforin (Fig. 2D) were higher in patients with high CDR3 scores. B lymphocyte-induced maturation protein-1 (Blimp-1) was reported to be highly expressed upon virus infection and play an important role in modulating the fate of effector T cells [19]. Compared with patients with low CDR3 scores, the mRNA expression levels of Blimp-1 in T cells were higher in patients with high CDR3 scores (Fig. 2E). T cell

Table 2
Clinical characteristics of patients in CDR3 low group and CDR3 high group.

Characteristic	CDR3 low (n = 15)	CDR3 high (n = 14)
Age (yr)		
Median	53.00	51.50
Range	43–66	22–70
Sex, n (%)		
Male	4(26.67)	7(50)
Female	11(73.33)	7(50)
Genotype, n(%)		
1b	10(66.67)	8(57.14)
2a	4(26.67)	6(42.86)
1a	1(6.66)	
Viral load		
Median	6.25E + 04	1.50E + 01
Range	1.50E + 01–5.88E + 03	1.50E + 01–1.78E + 07
ALT		
Median	30.00	26.50
Range	8–99	6–547
AST		
Median	27.00	32.00
Range	12–80	11–989
Lymphocyte (%)		
Median	32.90	31.90
Range	22.80–88.10	4.5–48.5
Lymphocyte ($\#10^9$)		
Median	1.30	1.35
Range	0.50–2.70	0.30–4.30

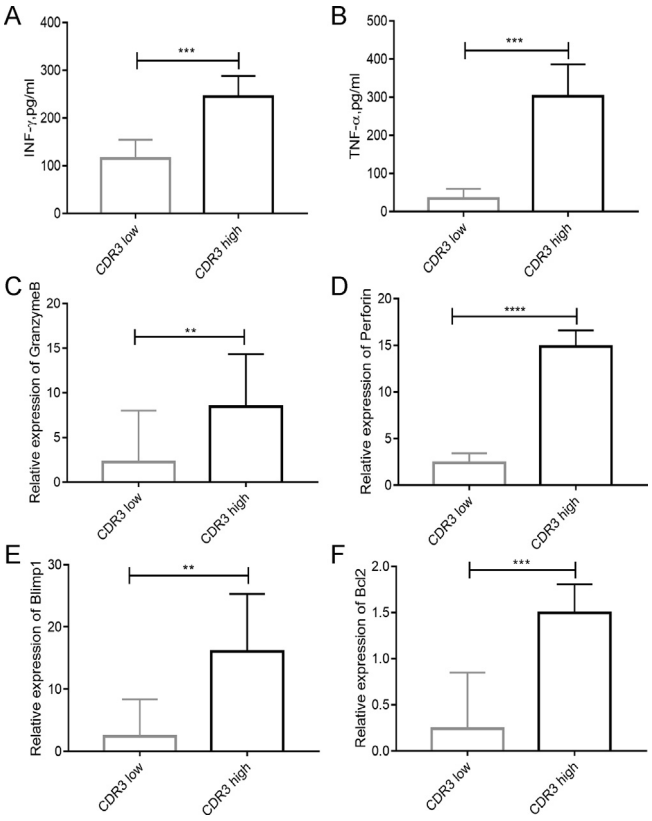

Fig. 2. The function of CD8+ T cells is robust in patients with high CDR3 scores. The levels of IFN-γ (A) and TNF-α (B) in patients' serum were detected by ELISA. Relative expression of GranzymeB (C), Perforin (D), Blimp1 (E) and Bcl-1 (F) were detected in patients with high CDR3 score and low CDR3 score by RT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

apoptosis is an important factor resulting in impaired T cell immune response in HCV patients. We further analyzed the relationship between B cell lymphoma/leukmia-2 (Bcl-2), a proto-oncogene that inhibited cell apoptosis, and CDR3 score in HCV patients. The mRNA expression of Blc-2 of T cells showed no difference in CDR3 high and low patients (Fig. 2F). These results suggested that CDR3 score is positively related to T cell function in HCV patients.

3.3. Low CDR3 scores were associated with poor prognosis in HCV patients

In clinic, good prognosis is the gold standard to evaluate the efficacy of antiviral therapy in HCV patients. We explored the relation of CDR3 score to the clinical prognosis indicators such as viral load (VL), rapid virologic response (RVR), early virologic response (EVR) and sustained virologic response (SVR). 5 patients were classified into SVR. 16 patients were classified into EVR, and 20 patients were classified into RVR. Patients with negative VL (≤1.50E + 01) showed higher CDR3 scores than patients with positive VL (> 1.50E + 01) (Fig. 3A). In contrast to patients that did not achieve RVR (nRVR), CDR3 scores of RVR patients (n = 20) were higher (Fig. 3B). Patients who achieved EVR (n = 16) also showed higher CDR3 scores compared to those did not achieve EVR (nEVR) (Fig. 3C). Indeed, CDR3 scores in patients achieved SVR (n = 5) were higher than those relapsed patients after antiviral therapy (Fig. 3D). These results revealed that patients with high CDR3 scores achieve better therapeutic efficacy, while low CDR3 scores are closely associated with poor prognosis in HCV patients.

3.4. Vβ27 played an important role in the clearance of HCV

As shown in Figs. 1 and 2, CDR3 scores were strongly associated with T cell function and prognosis. We further analyzed the expression profile of CDR3 subfamily members in healthy donors and HCV patients including recovery patients and antiviral therapy received patients. We found CDR3 subfamily members Vβ20, Vβ29, Vβ6, Vβ7-3 and Vβ27 ranked from 1 to 5 among all TCR subfamilies in recovery patients, and Vβ7-1, Vβ27, Vβ24, Vβ19 and Vβ9 were dominant subfamilies in HCV patients received ART (ART > 0 month) (Table 3). In addition, we analyzed Vβ families of last5 CDR3 score in HCV patient who didn't receive ART (ART = 0 month) and found Vβ17, Vβ23, Vβ27, Vβ10 and Vβ16 were the last5 subfamilies in these patients (Table 4). Interestingly, the CDR3 score of Vβ27 were significantly increased in both recovery patients (top 5) and patients with ART > 0 month (top 2). In contrast, Vβ27 score was significantly decreased in patients with ART = 0 month (last 3). It could be preliminary concluded that Vβ27 might play an important in the clearance of HCV.

4. Discussion

HCV infection-induced chronic infection can lead to liver fibrosis and carcinoma [14]. Immune responses mediated by virus-specific CD8+ T cells participated in HCV clearance or persistence, and therefore guiding the course of the disease. Numerous studies have confirmed that TCR-mediated CD8+ T cells were hypo-responsive in the liver of HCV-infected patients, which might be a key factor resulting in persistent infection and disease progression [20–23]. However, the underlying molecular mechanisms of CD8+ *T*-cell dysfunction in HCV patients have not been fully elucidated. TCR diversity was known to serve as a defining hallmark of the antigen-reactive T cell repertoire [24]. CDR3 was the most important region for the recognition of peptide-MHC complexes and represented the diversity of TCR repertoire [25]. We predicated that TCR diversity, particularly CDR3 spectratypes might determine the CD8+ T cell immune reactivity. In this study, we firstly isolated peripheral blood CD8+ T cells from twenty-nine HCV infected patients and healthy age-matched control donors and analyzed by RT-PCR for the expression of effector genes such as granzyme B, perforin and Blimp-1. Then, we analyzed the correlation of CDR3 scores with the expression of effector genes. The results showed that CDR3 score was positively related to T cell function in HCV patients.

The diversity of TCR has been recently reported a closely association with cancer prognostication [11,12,26]. A positive correlation between TCR repertoire normalization and remission of colorectal cancer was observed [11]. Higher tumor infiltrating lymphocyte (TIL) clonality correlated to improved survival among women with ovarian cancer [26]. *T*-cell repertoire in peripheral blood lymphocytes reversed to normal with a significant increase of *T*-cell repertoire complexity in hepatitis-associated aplastic anemia patients after successful treatment [12]. In our study, we found that CDR3 scores had a strongly relation to the clinical prognosis indicators such as VL, RVR, EVR and SVR. HCV patients with high CDR3 scores achieved better therapeutic efficacy, but those with low CDR3 scores had a poor prognosis. Therefore, monitoring the diversity of TCR repertoire might be helpful for determining the extent of immune system recovery and the degree of viral clearance in HCV-infected patients.

Conventional treatment regimen for chronic hepatitis C patients was the combination of IFN-α and ribavirin (RBV) several years ago [27]. Many direct-acting antiviral drugs such as telaprevir, boceprevir, sofosbuvir, simeprevir and daclatasvir have been launched in some Western countries for chronic hepatitis C patients [28,29].Unfortunately, these therapeutic strategies were only applicable for patients in chronic hepatitis stage. Up to now, there remains no effective therapeutic strategy for patients developing into liver fibrosis and carcinoma. New targets and novel therapies were needed to improve the outcomes of these patients.

It has been demonstrated that TIL repertoire could not be used as clinical biomarker because of its diversity in most tumors with no notable oligoclonal expansions [26], but some unique dominant TCRβ

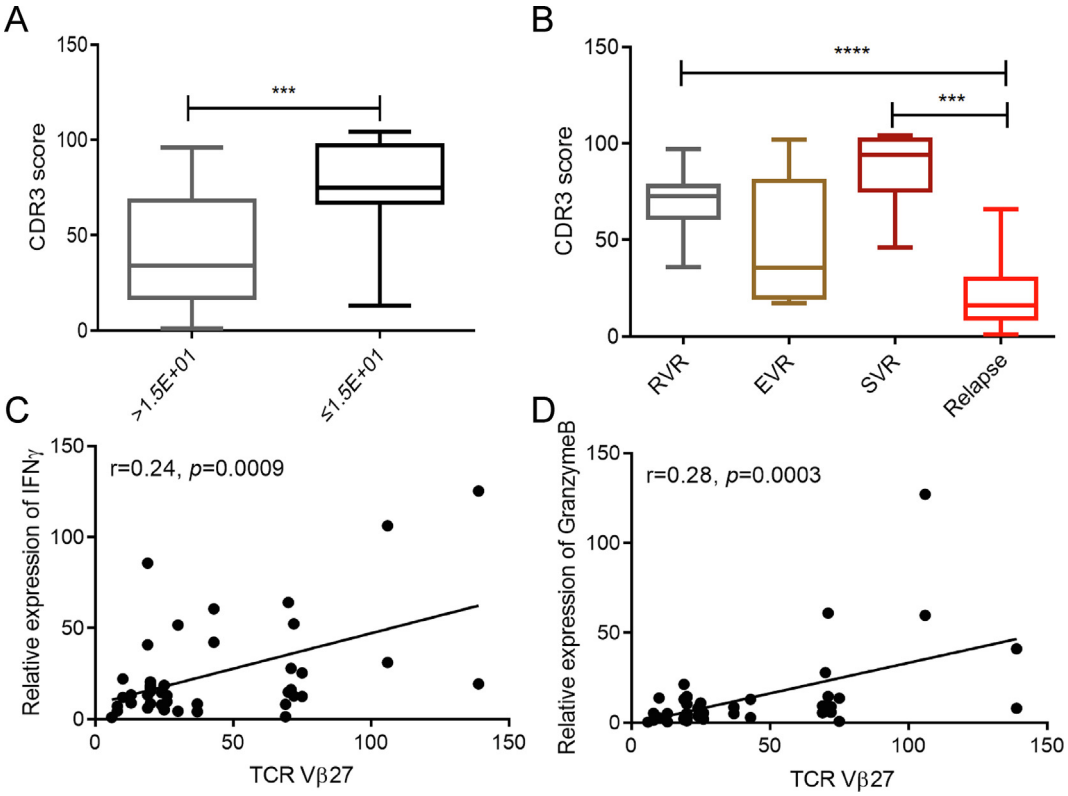

Fig. 3. Low CDR3 scores of CD8+ T cells is associated with poor prognosis in HCV patients. Shown were CDR3 scores of CD8+ T cells in HCV patients with (A) virus positive and negative group, (B) RVR and nRVR group, (C) EVR and nEVR group and (D) SVR and relapse group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3
Vβ families of top5 CDR3 score in different patient groups.

	HDs	Recovery patients	Patients (ART = 0 M)	Patients (ART > 0 M)
Top1	Vβ7-1	Vβ20	Vβ4	Vβ7-1
Top2	Vβ7-3	Vβ29	Vβ3	Vβ27
Top3	Vβ27	Vβ6	Vβ12	Vβ24
Top4	Vβ11	Vβ7-3	Vβ20	Vβ19
Top5	Vβ14	Vβ27	Vβ19	Vβ9

Table 4
Vβ families of last5 CDR3 score in different patient groups.

	HDs	Recovery patients	Patients (ART = 0 M)	Patients (ART > 0 M)
Last1	Vβ16	Vβ16	Vβ17	Vβ16
Last2	Vβ30	Vβ23	Vβ23	Vβ17
Last3	Vβ6	Vβ11	Vβ27	Vβ23
Last4	Vβ10-3	Vβ17	Vβ10	Vβ2
Last5	Vβ2	Vβ28	Vβ16	Vβ30

sequences emerged in patients with Refractory coeliac disease type II [13]. In our study, the CDR3 score of Vβ27 was found significantly increased in recovery patients (top5) and patients with ART > 0 month (top2) and significantly decreased in patients with ART = 0 month (last3). We preliminary speculated that Vβ27 might play an important role in the clearance of HCV and could be used as a therapeutic target for HCV patients. TCR-engineered T cells have emerged as a novel option of adoptive cell therapy for the treatment of cancer in recent decades [1]. T cells genetically engineered to express an oncoprotein-specific TCR from metastatic anal cancer patient's TILs displayed highly specific recognition and killing of human papillomavirus (HPV)+ epithelial tumor cells such as HPV-16+ cervical, and head and neck cancer cell lines [30]. Clinical study of TCR gene therapy targeting HPV-16 E6 for HPV-associated cancers has been finished (NCT02280811). Surprisingly, adoptive transfer of engineered hepatitis B virus (HBV) TCR-redirected T cells has been recently reported to supplement HBV-specific immune responses and facilitate HBV control in chronic HBV patients [31], indicating that engineered HCV TCR-specific T cells might be an effective strategy to overcome liver fibrosis and carcinoma in future. Based on the closely association between Vβ27 and the prognosis of HCV patients, we believed that engineered HCV Vβ27-specific T cells might be used as a novel option of adoptive cell therapy for the treatment of HCV-infected patients, particularly those with liver fibrosis and carcinoma.

In conclusion, patients with high CDR3 scores achieve better therapeutic efficacy, while low CDR3 scores are closely associated with poor prognosis in HCV patients. These findings indicate that TCR diversity maybe serve as a biomarker to predict the clinical parameters of HCV-infected patients.

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Conflicts of interest

The authors declare that there is no competing interest regarding the publication of this article.

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