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Original Article

Analysis of CDR3 polymorphism of T cell receptor by high-throughput sequencing in vitiligo patients

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Abstract: Objective: To investigate the CDR3 polymorphism of T cell receptor (TCR) in patients with vitiligo by high-throughput sequencing and immune repertoire analysis. Methods: A total of 45 patients with vitiligo were included in the case group, and 28 healthy volunteers were enrolled in the control group. Fasting venous blood collection was performed in both groups. Upon the total RNA extraction of cells, reverse transcription was carried out. Design and synthesis of downstream primers for TCR beta chain constant gene (TRBC) and specificity upstream primers for TCR beta chain variable gene (TRBV) gene family with common fluorescent labeling were conducted. Polymerase chain reaction was used for amplification of CDR3 in TRBV family. The CDR3 polymorphism and length of TRBV family were analyzed by means of a capillary electrophoresis genescan. Based on the analysis results, 8 samples in case group and 7 samples in control group were selected for cloning and sequencing of the TRBV6 family. The polymorphism, variable and joining gene expression as well as the composition, structure, and function of amino acid in CDR3 were also analyzed and the expression ratio of clonotype was detected. Results: Analysis of capillary electrophoresis genescan showed that compared with the control group, the CDR3 polymorphism in case group was significantly decreased, and the expressive abundance of TRBV20-1, TRBV7-9, and TRBV2 genes was the highest in the samples. The expression of TRBV7-9, TRBV25-1, TRBV4-1, TRBV12-5 showed significant differences, with the difference of TRBV7-9 being the most significant. The expressive abundance of clonotypes in different individuals differed greatly. There was no preference difference for amino acids within the groups, but there was an obvious difference in amino acid structure and function between the two groups. Analysis of a hyper-expressed clone found that the samples were highly heterogeneous, but the sequence similarity was very low, which proved that the expression difference of TRBV7-9 was the most obvious between the groups. Conclusion: The CDR3 polymorphism in patients with vitiligo was significantly increased.

Keywords: Vitiligo, high-throughput sequencing, CDR3, polymorphism

Introduction

Vitiligo, as the most common depigmenting skin disease in clinic, is mainly caused by selective destruction of melanocytes, and it infects both adults and children [1, 2]. At present, the pathogenesis of this disease is not completely clear yet. Genetic factors, biochemical factors, melanocyte auto-destruction and autoimmune disorder are important known causes [3, 4]. A clinical study confirmed that most patients with vitiligo are complicated with autoimmune diseases, which further suggests that vitiligo has a certain correlation with immune response disorder [5]. Once the immune response of human skin is imbalanced, specific damage often appears in melanocytes, which is another important cause of vitiligo [6]. T cells, which participate in the immune response, are vital for the pathogenesis of autoimmune diseases [7]. Mature T cells express the T cell receptors (TCR), and complementarity determining region 3 (CDR3) is able to reflect the polymorphism of TCR the most. CDR3 polymorphism also helps T cells recognize numerous antigens in human body. Therefore, analysis of TCR CDR3 sequence can further assist us in identifying T cell antigens [8]. High-throughput sequencing technology has been widely used in recognizing TCR CDR3 repertoires of autoimmune diseases and tumors [9]. However, it has not been used for vitiligo. Thus, this study aims to analyze the polymorphisms of TCR CDR3 in patients with vitiligo and to provide a reference for clinic.

Table 1. Sequence of primers

Name	Sequence (5'-3')
TRBV1-Sense	GCACAACAGTTCCCTGACTTGCAC
TRBV2-Sense	TCATCAACCATGCAAGCCTGACCT
TRBV3-Sense	GTCTCTAGAGAGAAGAAGGAGCGC
TRBV4-1-Sense	ACATATGAGAGTGGATTTGTCATT
TRBV5-1-Sense	TACTTCAGTGAGACACAGAGAAAC
TRBV5-2-Sense	TTCCCTAACTATTAGCTCTGAGCTG
TRBV6-Sense	AGGCCTGAGGGATCCGTCTC
TRBV7-Sense	CCTGAATGCCCCAACAGCTCTC
TRBV7-9-Sense	TTGGCGCGCATGGGCACCAGCCTCC
TRBV8-Sense	ATTTACTTTAACAACAACTTCCG
TRBV9-Sense	CCTAAATCTCCAGACAAAGCTCAC
TRBV10-Sense	CTCCAAAAACTCATCCTGTACCTT
TRBV11-Sense	TCAACAGTCTCCAGAATAAGGACG
TRBV12-5-Sense	AAAGGAGAAGTCTCAGAT
TRBV13-1-Sense	AAGGAGAAGTCCCCAAT
TRBV13-2-Sense	GGTGAGGGTACAACTGCC
TRBV14-Sense	GTCTCGAAAAGAGAAGAGGAAT
TRBV15-Sense	AGTGTCTCTCGACAGGCACAGGCT
TRBV16-Sense	AAAGAGTCTAAACAGGATGAGTCC
TRBV17-Sense	CAGATAGTAAATGACTTTCAG
TRBV18-Sense	GATGAGTCAGGAATGCCAAAGGAA
TRBV19-Sense	CAATGCCCCAAGAACGCACCCTGC
TRBV20-Sense	GCTCTGAGGTGCCCCAGAATCTC
TRBV21-Sense	GATTCACAGTTGCCTAAGGA
TRBV22-Sense	CAGAGAAGTCTGAAATATTCGA
TRBV23-Sense	GATCGATTCTCAGCTCAACAG
TRBV24-Sense	AAAGATTTTAACAATGAAGCAGAC
TRBV25-1-Sense	CAAAAGCCCTTCACAGACAT
TRBC Anti-Sense	TTCTGATGGCTCAAACAC
GAPDH Sense	AGGGGTCTACATGGCAACT
GAPDH Anti-Sense	CGACCACTTTGTCAAGCTCA

Materials and methods

Subjects

The whole blood of 45 patients with vitiligo (case group) was collected. Among them, there were 28 males and 17 females, aged 8-73 years, with an average age of 34±4.8 years and course of disease of 26±11.8 months. A total of 28 healthy volunteers were enrolled in control group, including 13 males and 15 females with an average age of 32±3.2 years. All vitiligo patients received none systemic treatment such as immunomodulatory drugs, photochemical therapy and corticosteroids in one month. The healthy controls did not have a positive family history of psoriasis or other autoimmune diseases. There was no significant difference in age and gender between the two groups. All fasting venous blood collected from patients and controls was delivered to the laboratory and processed within 4 hours. This study was approved by the Ethics Committee of The Eighth Affiliated Hospital, Sun Yat-sen University. Informed consents were obtained from all subjects and their family members.

ARM-PCR

Total RNA of blood was extracted, and its concentration was measured according to the operating instructions of kit. The cDNA template was synthesized by reverse transcription reaction in a polymerase chain reaction (PCR) amplification device according to the instructions of reverse transcription kit (TransGen Biotech, Beijing). PCR amplification was performed for the CDR3 of TCR beta chain variable gene (TRBV). The reaction system included: 2 μL of cDNA template, 2 μL of upstream and downstream primers for TRBV, 30 μL of purified water, 5 μL of 2 mM dNTP, 5 μL of 10 × buffer, 3 μL of $MgCl_2$ and 0.25 μL of Taq DNA polymerase. After 30 consecutive cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and 72°C for 8 min, it was stored at -20°C for standby application. The primers were synthesized by Bioengineering Co., Ltd. See **Table 1** [10].

DNA purification and recovery

Principal component analysis (PCA) was used to analyze the expression of the top10 genes, and the genes with the most obvious expression differences were selected for analysis. The PCR products in CDR3 of the TRBV7-9, TRBV25-1, TRBV4-1, TRBV12-5 family (with the most significant differences) were purified, recovered, concentrated, and amplified. They were purified by 3% agarose gel electrophoresis for 1 h. The target DNA strips of those gene families on the gel were removed by a clean, sharp scalpel, and the recovery of DNA fragment was conducted according to the instructions of gel extraction kit (CW0524S, KWBio, Beijing, China).

High-throughput sequencing on Roche 454 GS FLX

The amplified CDR3 adaptors were ligated to the receptor repertoire, and the linkers were

Table 2. Sequence analysis

Label	Case	Control	t	P
Total input sequences	24,260,922±11,092,587	19,924,033±11,657,515	1.593	0.115
Total good sequences	22,286,717±9,353,904	14,833,522±12,613,209	2.891	0.005
Clones	39,407±26,170	76,623±44,961	4.476	<0.001
Clones with stops %	7. 01±1.92	6.75±0.53	0.698	0.487
Clusterized clones %	75.32±20.32	67.39±10.67	1.905	0.060
V mappings found % of total	95.54±4.45	93.57±8.93	1.254	0.214
V not determined clones %	3.60±1.74	3.47±1.53	0.324	0.746
J mappings found % of total	93.85±4.79	92.42±9.60	0.846	0.400
J not determined clones %	0.18±0.28	0.13±0.05	0.933	0.353
Mapped LQ reads % of good	0.61±0.53	0.62±0.19	0.095	0.923
Out of frame clones	40.15±7.54	43.07±12.64	1.238	0.219
Sequencing information utilization %	92.50±5.00	89.23±10.33	1.814	0.073

Note: Total input sequences: pair-end 150 bp, successful Merge reads; total good sequences: CDR3 reads in total input sequences; clones: clone numbers; clones with stops: clonotype with CDR3 termination tag; clusterized clones %: MiTCR software was used for extracting CRD3, which clustered the resulting clonotype, some clonotypes with low quality and poor support would be clustered in the higher ones near them (more details refer to the MiTCR software instructions). V mappings found % of total: ratio of reads that could be aligned to V genes; V not determined clones %: the ratio of V genes could not be determined; J mappings found % of total: ratio of reads that could be aligned to J genes; J not determined clones %: the ratio of J genes could not be determined; mapped LQ reads % of good: the reads could be aligned to the order of magnitude; out of frame clones: clonotype number of outliers.

ligated to the DNA fragments by molecular biology techniques. The single-stranded DNA library was immobilized on DNA capture beads. Specific linkers were used to ensure the successful binding of the magnetic beads to the DNA fragments. Upon successful emulsification and dispersion by amplification reagents, the magnetic bead library formed fragments containing specific sample DNA and magnetic beads. Each fragment was independently amplified in its own microreactor without the influence of other sequences, and the amplification proceeded in parallel with multiple copies. Afterwards, the emulsion mixture was broken, and specific fragment of each magnetic bead was generated. The TRBV family CDR3 immune repertoire was successfully established.

Statistical analysis

SPSS 19.0 was used for data analysis. The t-test was used to compare the polymorphisms between the two groups, and the Mann-Whitney test was used to evaluate the differences of polymorphism among TRVB families. Peak Seanner Sofb, varev 1.0 software was used to analyze sequencing data. PCA was used to analyze the differences of principal components.

Results

Comparison of the reads and clone numbers in CDR3 between two groups

Based on the analysis of sequence data (**Table 2**), it was found that in the case group, the reads of Merge at CDR3 was significantly higher than that in the control group, while the clone number in the case group was significantly less than that in the control group, indicating that the clonotype polymorphism of the case group was significantly lower than that of the control group ($P<0.05$).

Comparison of expression quantity and categories of CDR3 clonotype between the two groups

The expression quantity and categories in CDR3 clonotype of both groups were shown in **Figure 1**. Compared with the control group, the expression quantity of clonotype in the case group increased significantly, but the category of clonotype decreased significantly. Therefore, there were high expression quantity and less categories of clonotype in the case group, demonstrating that the CDR3 polymorphism of the case group was significantly decreased compared with the control group.

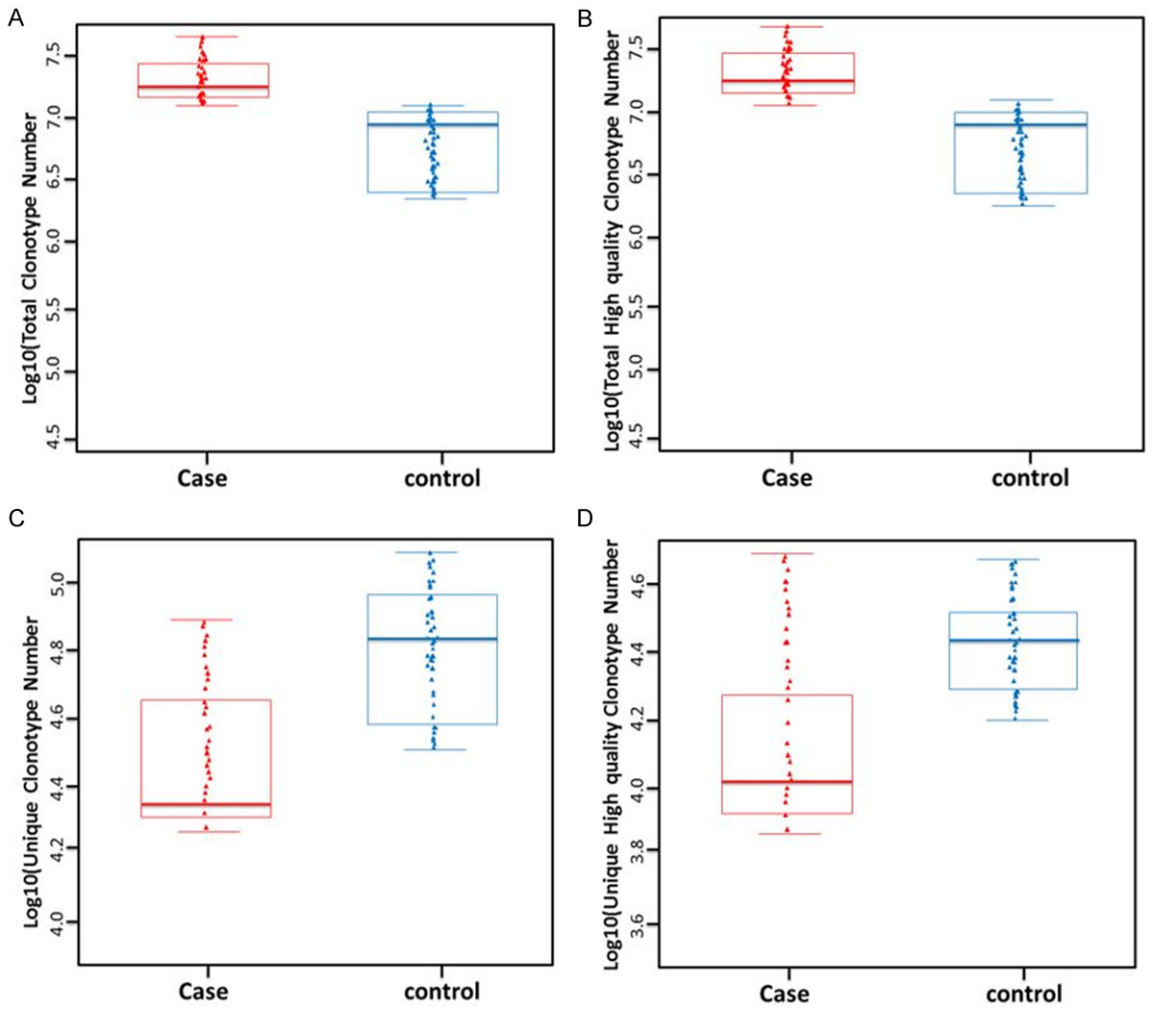

Figure 1. Analysis of clonotype expression quantity and categories of samples in two groups. A: Distribution of total clonotype number of each sample; B: Distribution of clonotype numbers with expression quantity more than 2; C: Distribution of total clonotype categories of each sample; D: Distribution of clonotype categories with expression quantity more than 2.

Analysis of CDR3 VJ gene

According to the expression richness analysis of CDR3 variable (V) and joining (J) genes, we found that the V and J genes in both groups had the same type of use, but the cumulative frequency was slightly different. TRBV20-1, TRBV7-9, TRBV2 expressed the most abundant among the samples (**Figure 2A** and **2B**). The distributions of the first principal component and the second principal component of the V gene were significantly different between the two groups (**Figure 2C**), indicating a significant difference in the compositional proportions. After the analysis of the expression of top10 genes, the main differences were found in the expression of TRBV7-9, TRBV25-1, TRBV4-1, and TRBV12-5, with the most significant difference in TRBV7-9 (**Figure 2D**).

Analysis of CDR3 amino acid

Analysis of amino acid composition, structure and function in CDR3 revealed that the length distribution of total clonotype was disordered, while the length distribution of unique clonotype was relatively uniform (**Figure 3A**), indicating that the expression abundances of clonotypes varied greatly among individuals. The difference might relate to different immune environments. The correlation coefficient between different samples was high with strong consistency (**Figure 3B**). The distribution of the amino acid composition ratio of clonotype

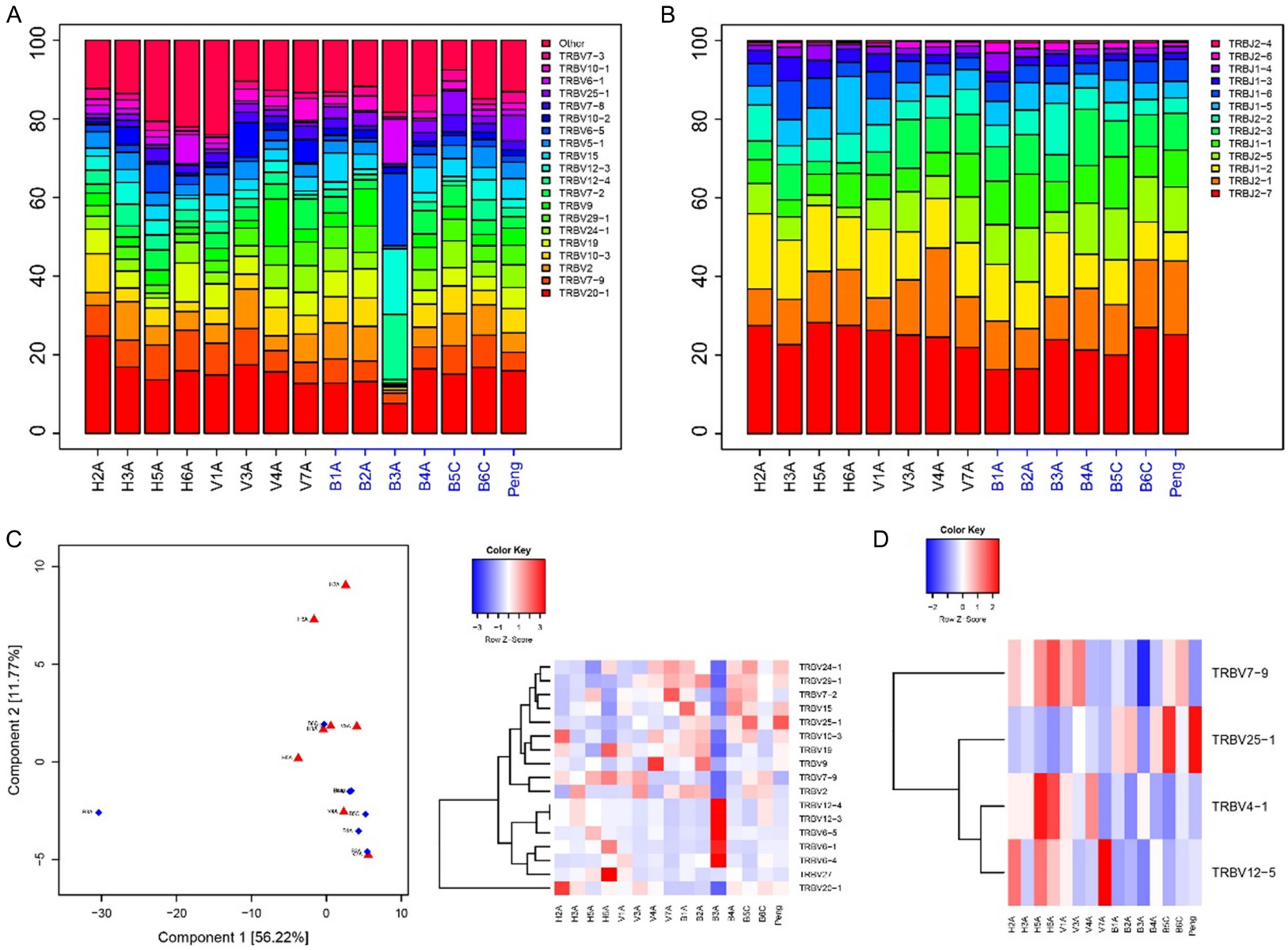
A
B
C
D
Other
TRBV7-3
TRBV10-1
TRBV6-1
TRBV25-1
TRBV7-8
TRBV10-2
TRBV6-5
TRBV5-1
TRBV15
TRBV12-3
TRBV12-4
TRBV7-2
TRBV9
TRBV29-1
TRBV24-1
TRBV19
TRBV10-3
TRBV2
TRBV7-9
TRBV20-1
TRBJ2-4
TRBJ2-6
TRBJ1-4
TRBJ1-3
TRBJ1-6
TRBJ1-5
TRBJ2-2
TRBJ2-3
TRBJ1-1
TRBJ2-5
TRBJ1-2
TRBJ2-1
TRBJ2-7
H2A
H3A
H5A
H6A
V1A
V3A
V4A
V7A
B1A
B2A
B3A
B4A
B5C
B6C
Peng
Component 1 [56.22%]
Component 2 [11.77%]
Color Key
Row Z-Score
TRBV7-9
TRBV25-1
TRBV4-1
TRBV12-5

Figure 2. Analysis of CDR3 VJ genes. A: Analysis of expression richness of V gene; B: Analysis of expression richness of J gene; C: Composition analysis of V gene; D: Heat map of differential V gene (t-test, $P<0.05$). The color shades represent the up-regulation (>0) or down-regulation (<0) of gene expression in each sample; vertical indicators indicate different gene families, and horizontal indicators represent different samples.

CDR3 showed that there was no significant difference in the amino acid composition ratio among the groups (**Figure 3C**), indicating that there was basically no preference for amino acids in different groups of samples. Cluster analysis found that the amino acids in control group and case group were significantly different in structure and function (**Figure 3D**).

Analysis of hyperexpressed clone

We tested the expression ratio of all clonotypes. The clonotype with a ratio of more than 1% per sample was considered to be a hyperexpressed clone. Analysis showed that the samples were highly heterogeneous, but the sequence similarity was very low (**Figure 4A**). Analysis of the expression of related sequence V genes showed that the expressions of TRBV7-9 were significantly different among groups (**Figure 4B**), so it was concluded that TRBV7-9 might relate to disease phenotype.

Discussion

Vitiligo as a common generalized depigmenting skin or acquired localized disease, appears at all parts of the body due to the dysfunction of skin melanocytes [11-13]. At present, studies have confirmed from the perspective of molecular immunity that T cells may be closely related to the pathogenesis of vitiligo. The mechanism may be that $CD4^+$ T and $CD8^+$ T cells are affected by surface-specific antigens of melanocytes, so their proliferation and activation are increased, and then, $CD4^+$ T and $CD8^+$ T secrete other cytokines that promote the proliferation of other immune cells and damage the melanocytes *in vivo* [14, 15]. The study of the mechanism of T cells in the pathogenesis of vitiligo is of great significance for the evaluation and treatment of this disease.

TCR presents on the surface of each T cell, and TCR3 is the most responsive to the diversity of TCR [16, 17]. Therefore, in this study, TCR CDR3 sequence was analyzed from the perspective of high-throughput sequencing. These results can help us better understand the pathogenesis of vitiligo and find a better target for the treatment of vitiligo. Understanding the polymorphism of TCR CDR3 repertoires and amino acids as well as the dynamic changes from a technical and theoretical perspective is the key to understanding the diversity of T cells [18, 19]. Comparison of the reads and clone number in the CDR3 between the two groups found that in the case group, the reads of Merge at CDR3 was significantly higher than that in the control group, while the clone number in the case group was significantly lower than that in the control group, indicating that the clonotype polymorphism of the case group was significantly lower than that of the control group ($P<0.05$), and the TCR CDR3 polymorphism in vitiligo patients was abnormal compared with the normal people. Analysis of the richness of VJ gene expression in CDR3 revealed that the gene expression differences between the two groups mainly appeared in TRBV7-9, TRBV25-1, TRBV4-1, and TRBV12-5, with TRBV7-9 being the most significant. Analysis of the amino acid composition, structure and function of the CDR3 showed that expression abundance of clonotype in different people varied widely. Sui et al. had the same finding when analyzing the amino acid composition in the CDR3 of TCR; this might relate to the different immune environment [20]. The cluster analysis showed that the amino acids in control group and case group were significantly different in structure and function, which might be the reason for the expression difference of the TCR CDR3 sequence between the two groups. Analysis of hyper-expressed clone of the related sequence V genes demonstrated that TRBV7-9 had the most significant difference between vitiligo patients and healthy controls, suggesting that TRBV7-9 might be associated with the disease phenotype.

In summary, polymorphism of TCR CDR3 in patients with vitiligo is significantly decreased, and TRBV7-9 may be associated with the disease phenotype. In this study, from the perspective of gene cloning, a new pathogenesis of vitiligo has been explained. However, there

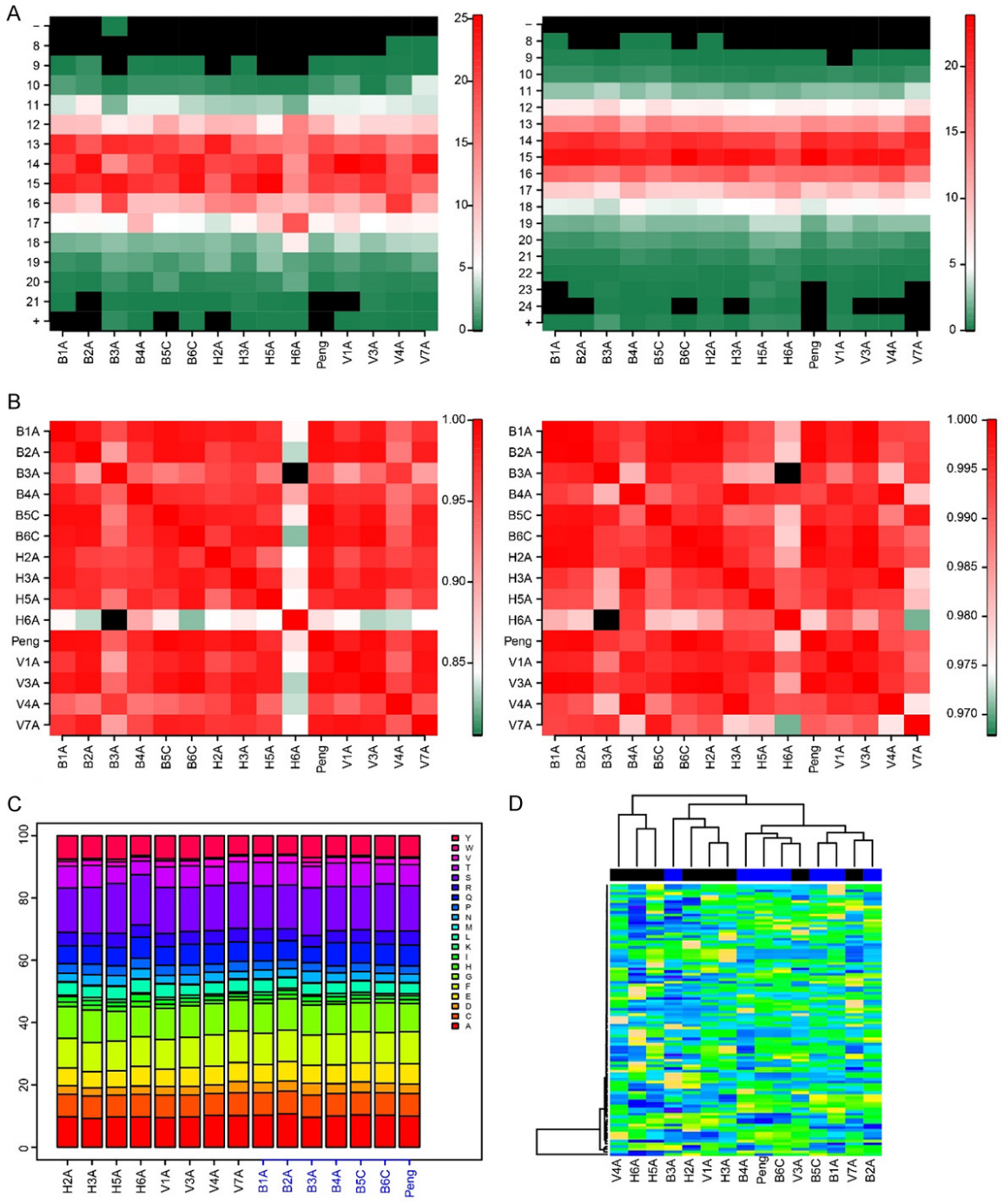

Figure 3. Analysis of CDR3 amino acid. A: Length distribution of total clonotype and unique clonotype; B: Distribution of sample correlations; C: Distribution of amino acid composition ratio of all clonotype CDR3; D: Structural and functional analysis of two groups.

are still some deficiencies in the study, such as the small number of cases and no real verification in a larger range of *in vitro* and *in vivo* experiments. Furthermore, the characteristics of the TCR CDR3 against antigens were not clarified, therefore it is difficult to clearly reveal the specific immune response mechanisms of vitiligo. It is believed that as the research on the TCR CDR3 polymorphism continues, clinical understanding of vitiligo will be clearer.

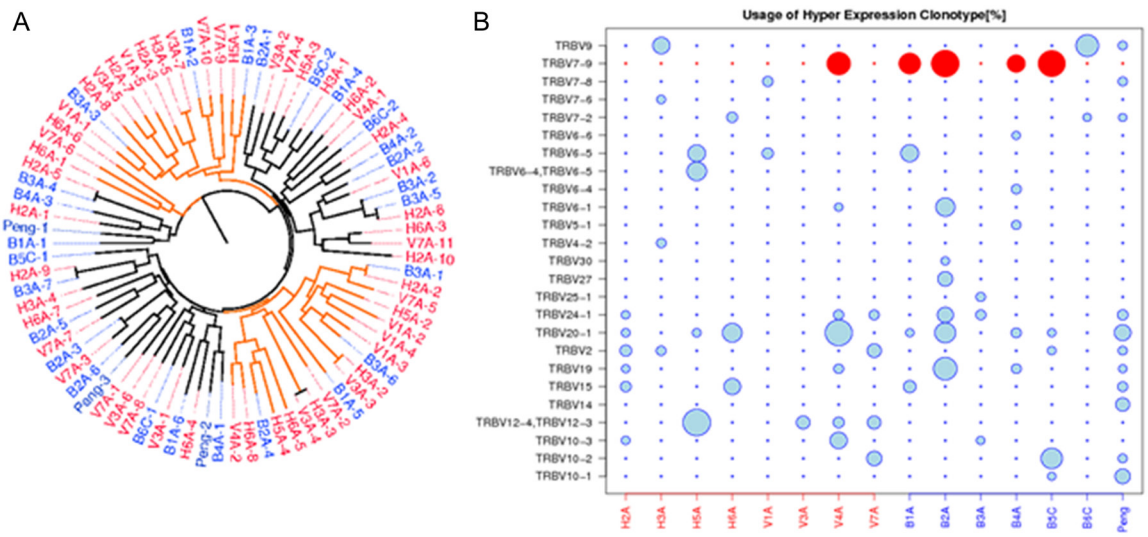

Figure 4. Structure similarity, expression similarity and V gene expression of hyper-expressed clonotype sequences. A: Analysis of the expression quantity of each sample on hyper-expressed clone; B: Analysis of V gene expression.

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Disclosure of conflict of interest

None.

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