Original Article
A primary study on T cell TCR β chain CDR3 spectral sequence detection in MSM HIV infected patients

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Abstract: The spectratyping of TRBV CDR3 have been applied in infectious diseases, autoimmune diseases and cancer. In this study, we used fluorescence quantitative PCR melting curve to analysis TCR CDR3 spectrum in peripheral blood samples of 41 healthy males and 33 sex with men (MSM) HIV infected patients. We aimed to evaluate the change in TCR β chain CDR3 and molecular features of HIV specific TCR TRBV family gene in the peripheral blood of HIV patients. Our results showed that MSM HIV infected patients had higher TRBV3, TABV7, TRBV9, TRBV20 and TRBV24 mono (oligo) expression rates compared with healthy male population. The difference was statistically significant by one-way ANOVA analysis (p<0.05). MSM HIV infected patients had higher TRBV subfamily miss rates (4.25%) than the healthy male control group (0.52%, chi-square test, $\chi^2=16.73$, p<0.05). We concluded that it is the first time in China to use the fluorescence quantitative PCR melting curve analysis to investigate the changes of TCR β chain CDR3 in HIV infected patients. Our results demonstrated that there was single (oligo) hyperplasia and deletion of the TCR BV family in MSM HIV infected patients, and there is statistically different TRBV distribution between MSM HIV infected patients and healthy population.

Keywords: HIV infection, TCR, CDR3, spectrum drift, fluorescence quantitative PCR melting curve analysis

Introduction
TCR CDR3 spectral patterns can well reflect the response and tolerance of T cells in infection, autoimmunity, and cancers. In recent years, the relationship between various viral infections and T cell response has been further clarified. Now specific TCR β chain CDR3 sequences have been found in HBV, HCV, EBV and other viral infectious diseases. Some results have been used in immune response mechanisms of the body to viruses, and to find out virus-specific T cell epitopes. Although it is known that abnormal changes occur in TCR CDR3 region in different periods of HIV-1 infection, the characteristics of TCR change at a specific period and in a particular course needs further study. Besides, there is no domestic report on the characteristics and application of TCR β chain CDR3 molecules of this group of T cells.

The technique of “fluorescence quantitative PCR melting curve analysis to monitor TCR CDR3 spectral drift in HIV-infected patients” has been established in our laboratory [1], we investigated the changes of TCR β chain CDR3 in the peripheral blood samples of 41 healthy males and 33 MSM HIV infected patients in Chongqing, to identify HIV-specific TCR TRBV families. In addition, we selected part of single/oligo-clonal proliferative T cells for CDR3 gene sequencing, to primarily investigate the changes in TCR β chain CDR3 spectrum. These findings suggested that TCR CDR3 spectral patterns is a useful method for studying T-cell immune response mechanism, disease process and personalized treatment in HIV infected patients.

Materials and methods
Main reagents and instruments
RNA extraction kit (Promega), cDNA synthesis and PCR reagents (Promega), lymphocyte separation medium (Promega), Realtime PCR Master
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Mix (TOYOBO), Fluorescence PCR instrument (Hangzhou Bioer FQ48), gel imaging system (ChemidocXRS), high-speed freezing centrifuge (Sigma 3K15), PAC300 electrophoresis apparatus (Bio-Rad), DG-3C electrophoresis tank (Beijing Ding Guo) and so on.

Primer design and synthesis

Primer design, sequence and synthesis are seen in literature [1]. 26 TCR BV gene family upstream primers, 1 TCR BC downstream labeled primer, and 1 control TRBC upstream primer were synthesized; the control GAPDH upstream and downstream primers (P1: cgaccttgtgcaagctca, P2: aggggtctacatggcaact) were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd.

Study subjects

Subjects were males aged from 18-40 years old. They were all from various districts and counties of Chongqing, native Chongqing Han people, and had no blood relationships. On inquiry, those with a family genetic history, autoimmune diseases, viral hepatitis and other recent infections were ruled out. Among them, the HIV infected patients were HIV/AIDS MSM people confirmed as HIV-1 antibody positive by Western Blot (WB) in Chongqing Municipal Center for Disease Control and Prevention AIDS Confirmation Center Laboratory, and receiving CD4+T lymphocyte monitoring in Chongqing Municipal Center for Disease Control and Prevention from 2012-2014, including past infection and newly discovered infection. A total of 33 cases were included, aged 18-40 years old, 32 years old on average. The 41 cases in the control group were healthy males from health examination, aged 18-38 years old, and 29 years old on average. Before blood sample collection, we got consent from all the subjects and investigated and gathered their basic information.

Lymphocyte separation, total RNA extraction and cDNA synthesis

Lymphocytes separation, total RNA extraction and cDNA synthesis (with OligdT as primers, reaction system 20 μl) were completed according to kit instructions. The cDNA synthesized was stored at -20°C for further use.

FQ-PCR amplification of the cDNA in TRBV family CDR3 segments

Reaction volume 20 ul. Reaction conditions: 94°C 3 min, 1 cycle; 94°C 30 sec, 55°C 30 sec, 72°C 50 sec, 45 cycles; 72°C 10 min 1 cycle. Melting curve analysis: 75°C~95°C, read fluorescence 0.2°C/s, 100 cycle. With GAPDH expression of red blood cells as the relative amount standard, calculate the relative expression of each TRBV family.

Criteria of TCR melting curve method CDR3 spectrum distribution and expression frequency

According to literature [2], in this study, on the peak gram of TCR melting curve CDR3 spectrum distribution and expression frequency, the standard of mono peak, oligo peak and no peak were as follows: “mono peak” (monoclonal): the emergence of a significant single peak, and its relatively “derivative (-dF/dT)” is greater than 85 or more; “oligo peak”: in non-standard Gaussian distribution family, there was a notable abnormal peak, the relative “derivative (-dF/dT)” is greater than 85; “no peaks” (deletion): there is no standard Gaussian, and no obvious peak, the relative “derivative (-dF/dT)” is less than 30.

Oligoclonal and abnormal distribution expression TRBV family selection and sequencing

According to criteria, count the “mono peak” (monoclonal), “oligo peaks” (polyclonal) and “no peak” (expression loss) of each family from TRBV1 to TRBV24. Data were statistically analyzed to find out the difference of TRBV family abnormal distribution and expression in MSM HIV infected patients. At the same time, the TRBV families with statistical difference were sequenced.

Relative expression amount analysis of each TRBV family

The GAPDH expression amount of the red blood cells in a healthy person was used as the relative amount standard to calculate the expres-
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Figure 1. Twenty-four TRBV family CDR3 melting curve spectral pattern in the PBMC of normal person N-1. TRBV1~TRBV24 were irregular multiple peak pattern. There was no phenomenon of mono peak, oligo peak or absence. TRBC and GAPDH for control were obvious mono peak pattern. The negative control had no melting curve peaks. Y-axis (vertical axis)-derivative (-dF/dT).

sion amount of each TRBV family. Analyze the expression situation of each TRBV family in MSM HIV infected patients and healthy people.
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**Statistical methods**

Statistical analysis was performed by one-way ANOVA analysis of variance with Tukey’s multiple comparison tests and non-parametric chi-square test by using the Statistical Package for the Social Sciences version 12.0 (SPSS). Two group differences were analyzed by

**Figure 2.** Twenty-six TRBV family CDR3 melting curve spectral pattern in the PBMC of HIV infected patient P-1. TRBV1–TRBV32 were mostly irregular multiple peak pattern. There were mono (oligo) peaks (TRBV3, TRBV6, TRBV7). TRBC and GAPDH for control were obvious mono peak pattern. The negative control had no melting curve peaks. Y-axis (vertical axis)-derivative (-dF/dT).
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Student’s t-test. The p value (two-tailed) of less than 0.05 was considered statistically significant.

Results

FQ-PCR amplification of TRBV family CDR3 segment cDNA

In the PBMC of most normal humans, the CDR3 spectral patterns of the 24 TCR β chains were multiple peaks. There was no mono peak, oligo peak or non-peak (eg. normal person-1, N-1). MSM HIV infected patients had oligo peak, partial peak and non-peak phenomena (eg. HIV infected patient-1, P1). The TRAC and GAPDH of a single product for control had mono peak pattern. The negative control had no melting curve peak (Figures 1, 2).

TRBV distribution and statistical analysis of MSM HIV infected patients and healthy population

In this study we investigated the expression of each TRBV family in 41 healthy Chongqing Han males and 33 MSM HIV infected patients. Among them, TRBV3, TABV7, TRBV9, TRBV20 and TRBV24 with monoclonal or oligoclonal expression had statistically different distribution in MSM HIV infected patients and healthy population (Table 1). The difference was statistically significant by chi-square analysis (p<0.05).

Relative expression amount and statistical analysis of each TRBV family in MSM HIV infected patients and healthy population

The GAPDH expression level of healthy human red blood cells was used as the relative amount standard. Fluorescence signals were collected to calculate the relative expression amount of each TRBV family in the subjects (results not shown). The relative expression amounts of the TRBV families who had statistically different abnormal expression in healthy population and MSM HIV infected patients in this study were statistically analyzed. Results showed that the relative expression amounts of each TRBV family with abnormal expression had no statistically significant difference (Student’s t-test, P>0.05).

Sequencing of monoclonal TRBV families in MSM HIV infected patients

TRBV3, TABV7, TRBV9, TRBV20 and TRBV24, which had higher abnormal expression rates in MSM HIV infected patients and statistically different distribution compared to healthy population, were sent to a sequencing company for sequencing. TRBV3 and TRBV24 were successfully sequenced (Sequence structure and function analysis published in another paper). The sequencing results of TRBV7, TRBV9 and TRBV20 indicate that this product is not a single product, and cannot be sequenced.

Discussions

The immune function of the body depends on the TCR antigen recognition function. For different antigens, the body can produce antigen specific TCR. According to the classical theory, when a gene is under positive selection, it is indicated that the population is in a new environment and the selection criteria has changed, needing new gene functions. When a gene is subjected to negative selection, the gene maintain its original function and tend to be stable [3]. Currently CDR3 spectrum drift detection in infectious diseases, especially viral diseases, has become a hot topic. Home and abroad, there are many reports on TCR Vβ CDR3 change and infectious diseases. Human cytomegalovi-
We used GAPDH expression amount of healthy males (0.52%). There is a significant difference between the two (χ² = 16.73, p<0.05). The results of this study also showed that the expression amount of each TRBV subfamily had no statistical difference between the healthy population and MSM HIV infected patients. Meanwhile, the relative expression amount of TRBV3, TABV7, TRBV9, TRBV20 and TRBV24 which had abnormal expression and statistical difference was not statistically different between the two monitoring groups. The possible reason is that the calculation of relative expression was based on the relative quantification of fluorescent signals. All the fluorescent signals that can be detected in the reaction tube were calculated, including the fluorescence signals of a variety of amplified products. The mono/oligo clonal expression amount (fluorescent signal) was not reflected.

The characteristic of TCR variable region disorder in HIV-1 infected patients is the occurrence of non-normal distribution of different length CDR3 sequences, and significantly reduced diversity. This phenomenon has been confirmed in a number of studies. The study of Kou et al. found out in eight HIV-1 infected patients that the expression of TRBV2, TRBV3, TRBV7, TRBV9, TRBV20 and TRBV24 which had abnormal expression and statistical difference was not statistically different between the two monitoring groups. The possible reason is that the calculation of relative expression was based on the relative quantification of fluorescent signals. All the fluorescent signals that can be detected in the reaction tube were calculated, including the fluorescence signals of a variety of amplified products. The mono/oligo clonal expression amount (fluorescent signal) was not reflected.

At present studies have shown that HIV is closely related to T cell response and individual MHC type, but there have been no perfect HIV research animal model, systemic research methods and assessment tools. The TCR αβ T cell CDR3 length immune spectrum analysis technique we establish is a relatively reliable and simple way to analyze specific T cells. It is able to find single/oligoclonal proliferation T cells in a sample, provide composition information of T cell response library (partial peak and expression frequency change in different families), and dynamically monitor TCR change. To investigate the relationship between peripheral blood clonal proliferative T cell and HIV and its application, and to avoid information deviation from individual testing, we used Chongqing Han healthy males as the control, and test the PBMC of Chongqing Han MSM HIV infected patients for complete TCR BV CDR3 spectrum analysis. Results showed that in MSM HIV infected patients, the TCR BV families had mono (oligo) proliferation and absence phenomena, increased non-normal distribution, and decreased diversity. In MSM HIV infected patients, the mono (oligo) expression frequency of TRBV3, TABV7, TRBV9, TRBV20 and TRBV24 was higher than that in healthy male population. The difference was statistically significant by one-way ANOVA analysis (p<0.05). The RT-PCR products of the TRBV subfamilies above were sequenced, and results found out that some mono (oligo) hyperplasia expression TRBV products are mixtures of the same size (or a difference of about 3 bp leading to different sequence) instead of a single product. This phenomenon has also been reported abroad [6]. The investigation also found that MSM HIV infected patients had higher TRBV subfamily miss rate (4.25%) than the control group of healthy males (0.52%). There is a significant difference between the two (χ² = 16.73, p<0.05). We used GAPDH expression amount of healthy human red blood cells as the relative amount standard, and collected fluorescence signals to calculate the relative expression amount of each TRBV family. However, results showed that the expression amount of each TRBV subfamily had no statistical difference between the healthy population and MSM HIV infected patients. Meanwhile, the relative expression amount of TRBV3, TABV7, TRBV9, TRBV20 and TRBV24 which had abnormal expression and statistical difference was not statistically different between the two monitoring groups. The possible reason is that the calculation of relative expression was based on the relative quantification of fluorescent signals. All the fluorescent signals that can be detected in the reaction tube were calculated, including the fluorescence signals of a variety of amplified products. The mono/oligo clonal expression amount (fluorescent signal) was not reflected.

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used in advantages of the individual patient, and some individuals also had a variety of TCR AV and TCR BV families of mono/oligoclonal expression at the same time (such as the HIV infected patients numbered p6 and p8). This may be associated with different HLA phenotypes of the individual and diversity of HIV antigens.

When the same virus infects different population, there is different family predomination utilization (TRBV, TRBJ and TRBD). This suggests T cell bias, and is closely related to the diversity of HLA and virus CTL epitopes. The change and predominant expression of TCR Vβ family are different in HIV infected patients. This may be associated with different HLA in different ethnicity and regions, as well as the diversity of virus CTL epitopes. It may also due to that the single antigen epitope can induce clonal proliferation of T lymphocytes of different TCR CDR3 [14]. Further researches are required to determine whether TCR B-chain-specific shift in HIV and other viral infections is due to predominant expression after virus infection. Our study suggested that the technique of the fluorescence quantitative PCR melting curve analysis have potential as a diagnosis and therapeutic approach to monitor TCR β chain and CDR3 subfamily growth for HIV infected patients in clinical application.

Disclosure of conflict of interest
None.

Abbreviations

PCR, polymerase chain reaction; TCR, T cell receptor; CDR3, third complementarity region; TRBV, T-cell receptor beta-chain variable.

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