

Comparison of Two Methods for Screening TCR V β Biases of T1DM Patients: Silver Staining for Polyacrylamide and Ethidium Bromide Staining for Agarose Gel Electrophoresis

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Abstract Objective: To explore a fast and sensitive method for screening T cell receptor (TCR) biases through comparison of silver staining for polyacrylamide gel electrophoresis and ethidium bromide staining for agarose gel electrophoresis. **Methods:** The lymphocytes were isolated from peripheral blood which collected from ten patients with T1DM; the total RNA was extracted followed by transformed to cDNA, and then electrophoresis was carried out on polyacrylamide and agarose gel simultaneously, finally stained with silver and ethidium bromide dyeing respectively. **Results:** The pictures of TCR V β biases detected with silver staining for polyacrylamide gel electrophoresis were very similar to that with ethidium bromide staining for agarose gel electrophoresis, V β 7, V β 11 and V β 17 were the mainly biased genes with higher frequencies, 10 and V β 13.2 were the common genes which did not biased in all the ten patients. In some V β genes, the number of the bands detected with the former technique was more than that with the latter methods. So did the clarity of some bands. **Conclusions:** Silver staining for polyacrylamide gel electrophoresis is a fast, sensitive and safe method for screening the biases of TCR V β genes.

Keywords: silver staining, polyacrylamide gel electrophoresis, ethidium bromide staining, agarose gel electrophoresis, T cell receptor, *typ1 a diabetes mellitus*

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1. Introduction

Mature T cells express one of two types of TCR: a heterodimer of α and β chains or γ and δ chains. High expansions of $\alpha\beta$ T cells within the TCR repertoire have been shown to occur in various malignancies and immunological disorders, in addition to inflammatory and infectious diseases [1]. If biased TCR variable region (V) was associated with the pathogenic T-cell populations, the corresponding TCR elements could be targeted by TCR-specific immunotherapies, such as TCR peptide or T-cell vaccination [2,3]. For this reason, the studies of TCR V gene biases are attached much weight by researchers.

To date, the techniques, such as Southern blot analysis [4], staining with TCR V-gene-specific antibodies [5], PCR-ELISA [6], immuoscope spectratyping technique [7] and so on, were applied to detecting expansion of TCR V gene. More presently, the real-time florescence quantitative polymerase chain reaction (FQ-PCR) and DNA melting curve analysis technique was successfully modified and

used to detecting skewness of TCR [8,9]. However, the above methods either need special instruments, or expensive reagents, or the procedure was too complicated to operate. Although there had been a method, ethidium bromide staining for agarose gel electrophoresis (which is called ethidium bromide staining technique in the following text), to screen the skewness of TCR, but its poisonousness of the dyeing limits its application. In this study, we used silver staining for polyacrylamide gel electrophoresis (which is called silver staining technique in the following text) and ethidium bromide technique to detect biases of TCR V β of patients with type 1 diabetes mellitus (T1DM), and hope to ensure if the former method could replace the later one.

2. Methodology

2.1. Subjects

All the patients with T1DM were diagnosed according to the standard of WHO [10]. Peripheral blood

lymphocytes (PBL) were collected from ten patients with T1DM and healthy volunteers, who were not treated with immunomodulating drugs in the previous six months prior to the study and were seronegative for markers of hepatitis viruses, HIV and other pathogenic infections. Excluded from the study were patients with tumors and immunological disorders. This study protocol was approved by the hospital Ethics Committee.

2.2. Extraction of RNAs and Synthesis of the First cDNAs

The sense primer, anti-sense primer and specific primers for 24 TCR Vβ genes families (both of Vβ5 and Vβ13 include two subfamilies: Vβ5.1 and Vβ 5.2, Vβ13.1 and Vβ13.2) were previously described [1] and synthesized by the Guangzhou Daangene Corporation of China. A total of 5 ml of blood were taken from each of T1DM patients and healthy volunteers respectively. PBLs were isolated by Fcoll-Hypaque density centrifugation. Using an Omega RNA extraction kit according to the manufacturer's instructions, total RNA was extracted, and 1 μg total RNA was reverse transcribed with 250 pm olig (dT), 200 U Moloney murine leukemia virus (M-MuLV) reverse transcriptase, and 2 μl of 10 mM dNTP mix (cDNA Synthesis Kit; MBI-Fermentas), in a total volume of 20 μl (six reactions for every sample). The cDNA was stored at -80°C.

2.3. Analysis of TCR Vβ Clonality with Two Methods

Ethidium bromide staining for agarose gel: according to the previous study [1,8,9], 8 μl PCR products were added in 1.5% agarose gel followed by staining with ethidium bromide.

Silver staining for polyacrylamide gel electrophoresis: 2 μl PCR products was spotted in 12% polyacrylamide gel with the thickness of 1 millimeter, and performed electrophoresis for 60 min, and then stained with 0.2% silver nitrate for 30 min, developed by 3% sodium hydroxide and 0.1% formaldehyde.

3. Results

On the 1.5% agarose gel stained with ethidium bromide, each family of TCR Vβ genes of healthy volunteers only showed a comparatively clear band at the position of about 250 bp, and the picture of every Vβ gene was similar to that of Vβ1 of patient-1 (as shown as Figure 1), so the data was not shown. While for the patients with T1DM, except for most Vβ genes exhibited single clear band at the predicted position, several showed two or more than two bands, or there were a few genes which exhibited more blurry bands, even looked disappear (Figure 1, only the picture of patient-1 was shown). Compared to healthy volunteers, except for Vβ8, Vβ10, Vβ12, Vβ13.2 and Vβ15, the other Vβ genes showed more or less biased, and the mainly biased genes were Vβ7, Vβ11 and Vβ17. Besides, the bands of Vβ18 of patient-1, Vβ20 of patient-6 and -8 were found deficient or deleted (Table 1).

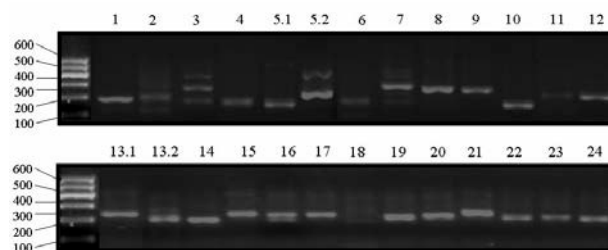


Figure 1. The pictures for 24 TCR Vβ genes of T1DM patients-1 on the 1.5% agarose gel by ethidium bromide staining

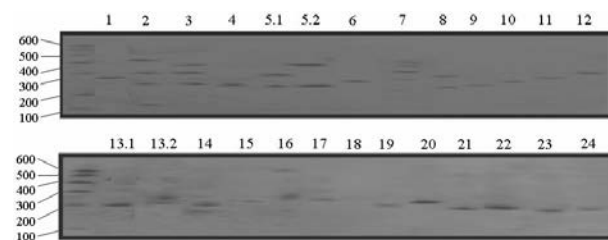


Figure 2. The pictures for 24 TCR Vβ genes of T1DM patients-1 on the 12% polyacrylamide gel by silver staining

Table 1. The biases of 24 TCR Vβ genes in T1DM patients with agarose gel electrophoresis by ethidium bromide staining

Vβ	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	A	A	A	A	B	A	A	B	B	A
2	B	A	A	B	A	A	A	A	A	B
3	C	A	A	A	A	A	A	A	A	A
4	A	A	A	A	A	A	A	A	A	A
5.1	A	A	A	A	A	A	A	C	A	A
5.2	B	A	A	A	A	A	A	A	A	A
6	A	A	A	A	A	A	C	A	A	A
7	D	B	A	A	B	B	A	A	B	C
8	A	A	A	A	A	A	A	A	A	A
9	A	A	B	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A
11	A	B	C	A	C	B	A	A	A	A
12	A	A	A	A	A	A	A	A	A	A
13.1	A	A	A	A	A	A	A	B	A	A
13.2	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	B	A	A	A	A	A
15	A	A	A	A	A	A	A	A	A	A
16	A	A	A	A	C	A	A	A	A	A
17	A	B	B	B	A	A	A	A	A	B
18	▲	A	A	A	A	A	B	A	A	A
19	A	A	B	A	A	A	A	A	A	A
20	A	A	A	A	A	▲	A	▲	A	A
21	A	B	A	A	A	A	A	A	A	A
22	A	A	A	A	A	A	A	A	B	A
23	A	A	A	A	B	A	A	A	A	A
24	A	A	A	A	A	A	A	B	A	A

A, B, C, D and E represented one, two, three, four and five band(s) respectively; ▲ represented the TCR Vβ gene families which exhibited no band.

Table 2. The biases of 24 TCR Vβ genes in T1DM patients with polyacrylamide gel electrophoresis by silver staining

Vβ	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	A	A	A	A	B	A	A	B	A	A
2	D	A	A	A	A	A	A	A	A	B
3	C	A	A	A	A	A	A	A	A	A
4	A	A	A	A	A	B	A	A	A	A
5.1	B	A	A	A	A	A	A	C	A	A
5.2	B	A	A	A	A	A	A	A	A	A
6	A	A	A	A	A	A	D	A	A	A
7	E	C	A	D	B	B	A	C	B	C
8	B	A	A	A	A	A	A	A	A	A
9	A	A	B	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A
11	A	C	D	A	C	B	A	A	A	C
12	A	A	C	A	A	A	A	A	A	A
13.1	A	A	A	A	A	A	A	B	A	A
13.2	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	D	A	A	A	A	A
15	A	A	B	A	A	A	A	A	A	A
16	B	A	A	A	A	A	A	A	A	A
17	A	B	D	B	A	B	A	A	A	C
18	▼	A	A	A	A	A	B	A	A	A
19	A	A	B	A	A	A	A	A	A	A
20	A	A	A	A	▼	A	A	▲	A	A
21	A	C	A	A	A	A	A	A	A	A
22	A	A	A	B	A	A	A	B	A	A
23	A	A	A	A	C	A	A	A	A	A
24	A	A	B	A	A	A	A	B	A	A

A, B, C, D and E represented one, two, three, four and five band(s) respectively; ▲ represented the TCR Vβ gene families which exhibited no band; ▼ represented the TCR Vβ gene families which exhibited very blur band.

On the 12% polyacrylamide gel stained with silver solution, each picture of Vβ graph of the healthy volunteers also showed a thin band at about the position of 250bp, and all like the picture of Vβ4 of patient-1 (as shown as Figure 2), so the data was not shown as well. While for the patients with T1DM, except for most Vβ genes exhibited single clear bands at the predicted position, several showed two or more than two bands, or there were a few genes which exhibited more blurry bands, even looked disappear (Figure 2, only the graph of patient-1 was shown). Compared to healthy volunteers, except for Vβ10 and Vβ13.2, the other genes showed more or less skewed, the mainly biased genes also were Vβ7, Vβ11 and Vβ17, however, the bands of Vβ18 of patient-1 and Vβ20 of patient-6 showed more blurry, that of Vβ20 of patient-8 was deficient or deleted (Table 2).

Comparatively, the pictures attained by the silver staining technique were similar to that of the ethidium bromide one, however, the clarity of the bands of all the samples were different for two methods, it was better for the former than that of the latter; the number of the bands of several TCR Vβ were not completely identical, generally, the number of the bands with the former technique was high than that with the latter one. For example, there were two and four blur bands in Vβ2 and Vβ7 respectively in T1DM patient-1 detected by ethidium bromide staining technique, while by silver staining technique, there were four clear bands for Vβ2, while the number for Vβ7 was five (Figure 1 and Figure 2). We detected some of the Vβ families which exhibited more than two bands with the two methods, and found that the number and/or clarity of the bands assayed by silver staining technique were significantly higher and/or better than that by ethidium bromide one (Figure 3).

4. Discussion

Ethidium bromide staining technique is a classic method to detect expression of the RNA or DNA; recently,

it has been used to quickly detect the clonality of TCR [11]. In the previous studies, we used the technique to detect the skewness of TCR Vα or/and Vβ genes of healthy human, patients with colorectal carcinoma, childhood patients with tuberculosis infections and children vaccinated with Bacillus Calmette-Guerin, and described the normal genes of health volunteers and biased gene families of some patients [1,8,9], moreover, we found that the results detected with ethidium bromide staining technique were coincident with that with genescan or DNA melting curve analysis technique. This method was more quick and simple, so it can be taken as a proper technique to screen the skewness of TCR. However, because of its toxicity, its application is limited to some degree.

Silver staining technique is a sensitive method for detecting relative quantity of protein, enzyme, RNA, DNA and so on, which detectable range could be as low as the level of nanogram [12,13]. However, to date, if it can be used to detect the biases of TCR V genes remain unknown. In this study, we used the silver staining and ethidium bromide staining technique to assay the TCR Vβ biases of T1DM patients simultaneously, and found that the TCR Vβ pictures of all ten patients detected with the former method were very similar to that of the latter one. Vβ7, Vβ11 and Vβ17 were the mainly biased genes with higher frequencies, while Vβ10 and Vβ13.2 were the common genes which showed were not biased in all of the ten T1DM patients. There were also differences between the results detected by the two methods respectively. Firstly, in some TCR Vβ genes of the same patients, the number of bands attained by silver staining technique was higher than that detected by ethidium bromide staining technique, such as Vβ2 and Vβ7 of patient-1, in the former method, the number were four and five respectively, while that were only two and four in the latter one. This indicated that silver staining technique could isolate the DNA more exactly than ethidium bromide staining technique. Secondly, the bands, especially besides the ones of about 250 bp, were much

clearer with silver staining technique compared to that with ethidium bromide staining way, for example, at location of about 300bp, the band of TCR V β 7 of patient-5 assayed by the former method was significantly obvious than which by the latter method (as showed in Figure 3). Thirdly, except for the electrophoresis instrument and staining reagents, silver staining technique needs no other equipment, while ethidium bromide technique needs the ultraviolet transilluminator to observe the results. Lastly, ethidium bromide dyeing is poisonous, and the ultraviolet ray is another harmful factor to researchers, while all of the instrument and reagents in the silver staining technique are safe to human user.

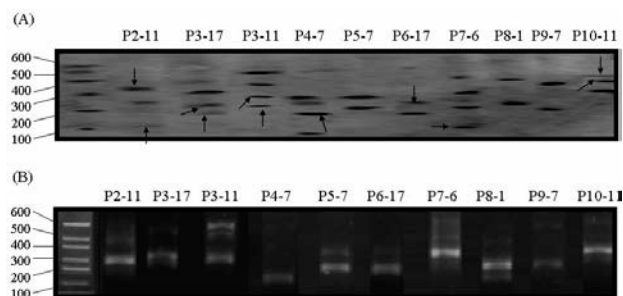


Figure 3. The comparative pictures for some of TCR V β genes of T1DM patients which showed biased on 12% polyacrylamide gel by silver staining and on the 1.5% agarose gel by silver staining respectively. The number such as P2-11 on the figure represented as follows: P2-11: V β 11 of patient-2, P3-17: V β 17 of patient-3, P3-11: V β 11 of patient-3, P4-7: V β 7 of patient-4, P5-7: V β 7 of patient-5, P6-17: V β 17 of patient-6, P7-6: V β 6 of patient-7, P8-1: V β 1 of patient-8, P9-7: V β 7 of patient-9, P10-11: V β 11 of patient-10. (A) The graphs for some of TCR V β genes of T1DM patients which showed biased on 12% polyacrylamide gel by silver staining. (B) The graphs for some of TCR V β genes of T1DM patients which showed biased on the 1.5% agarose gel by ethidium bromide staining

However, the small sample size, the concentration of polyacrylamide gel and the relative complexity of the polyacrylamide gel electrophoresis are the main elements which affect the detection effect of silver staining technique; in future, we will use more cases and more appropriate conditions to explore the usage of the method in the fields of TCR skewness.

5. Conclusion

Overall, compared with ethidium bromide staining technique, silver staining is a quick, safe and effective method to detect the clonotypes of TCR V β gene families of T1DM patients, it is worthy to consider as a screening assay to evaluate the bias of TCR V β .

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