Article

A Simplified PCR-SSP Method for HLA-A2 Subtype in a Population of Wuhan, China

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HLA-A2 is the most frequent HLA-A allele in all ethnic populations, and an important restriction element for peptide presentation to T cells in infectious disease and cancer. However, the HLA-A2 supertype consisting of up to 75 subtypes, mutation studies and analyses using cytotoxic T lymphocytes suggest the functional relevance of subtype-specific differences in HLA-A2 molecules for peptide binding and T-cell recognition. Therefore, it is necessary for T-cell response study to discriminate the HLA-A2 subtypes and to understand the profile of HLA-A2 allelic distribution in a given population. In this study, we developed a simple, robust approach based on the nested polymerase chain reaction using sequence-specific primers (PCR-SSP) to discriminate 17 HLA-A2 subtypes which cover the most HLA-A2 alleles (> 99% allele frequency) reported in Chinese, using 15 combinations of 19 allelic specific primers. In the first round of PCR, 3 combinations of 5 primers were used to determine whether the tested sample was HLA-A2 positive, meanwhile the subtypes of HLA-A*0209 and HLA-A*0215N were determined for the variant position of these two subtypes is in exon 4 instead of exon 2, 3. Samples of HLA-A2 positive were subtyped in the second round of PCR, using PCR products of the first round as templates. This strategy was applied to test the samples of 78 random HLA-A2 positive individuals for their HLA-A2 subtypes. Those samples were screened for HLA-A2 positive by the first round PCR-SSP from 154 healthy blood donors in Wuhan, China. The subtyping results were verified by using flow cytometric analysis (FCM) with HLA-A2 specific monoclonal antibody BB7.2 and DNA sequencing. The typing results of the samples show 50.7% random individuals in the population carry HLA-A2, HLA-A*0201 ranks the first (allele frequency = 15.5%), followed by A*0207 (5.8%), A*0206 (4.7%), A*0203 (2.6%), A*0210 (0.7%), and these 5 alleles account for 99.0% HLA-A2 subtypes of allele frequency. Our study indicates that the developed typing method is simple and reliable for HLA-A2 subtyping in Chinese, and the profile of allelic distribution of HLA-A2 subtypes is revealed in the population of Wuhan, China. ***Cellular & Molecular Immunology*****. 2006;3(6):453-458.**

Key Words: HLA-A2, PCR-SSP, subtype

Introduction

Human lymphocyte antigen (HLA)-A2 is the most frequent HLA-A specificity in the human population with an allele frequency of 10-40% in different ethnic groups (1). It is also the most heterogenous HLA-A specificity with at least 75 subtypes identified to date (2). All of these subtype polymorphisms are almost located in exon 2 and exon 3 except HLA-A*0209 and HLA-A*0215N. Since these two exons encode the α1 and α2 domains that form the peptide binding groove of the major histocompatibility (MHC) class I and are responsible for peptide binding, antigen presentation and HLA restricted recognition by T-cell receptor (3). These subtypes differ from each other by one or a few amino acids, and these amnio acid changes have profound effect on the peptide binding characteristic as well as on the fine specificity of many HLA-A2-restricted cytotoxicity T-lymphocyte clones (3-6). Moreover, HLA-A2 subtype mismatch is reported to be able to elicit strong allogeneic responses in unrelated bone marrow transplantation (7, 8). Classical typing by serology can best identify three HLA-A2 subtypes (A*0203, A*0210, A*0217), clustering HLA-A*02 in a group including up to 75 subtypes and to get the subtype specificity monoclonal antibody is very difficult (2). Allele

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sequence-specific primers (SSP)-based polymerase chain reaction (PCR) technologies (PCR-SSP) have enabled the progress of typing for HLA class I and class II alleles. A high resolution DNA typing method for discriminating as many HLA-A2 alleles and typing in different ethnic and regional populations would have great implications for organ transplantation, population genetics and disease association studies. As population studies continue, the number of newly discovered HLA-A2 alleles is continuously growing so that eventually more and more primers may suffice for all their discrimination, but that is making the subtyping not cost-effective (2). Based on the HLA-A2 alleles reported in various Chinese populations, the most frequent HLA-A2 alleles are A*0201, A*0203, A*0206, A*0207 and A*0210, which account for > 99.0% (allele frequency) in Chinese (5). Therefore, we established a simplified, nested PCR-SSP HLA-A2 allele typing method using 15 combinations of 19 allelic specific primers, which was applied to investigate the profile of HLA-A2 allelic frequency in a population of Wuhan, China.

Materials and Methods

Population samples and reference cell line

A total of 154 individuals, who were healthy, unrelated and genetically Chinese blood donors from the Wuhan Red Cross Blood Center, were included in the present study. Genomic DNA was extracted from Natrium Citricum anticoagulant 500 μl of whole blood by the standard salt fractionation and ethanol precipitation. PCR-SSP works well for a range of DNA concentration 80-300 ng/ml. Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Hypaque density gradient centrifugation for FCM assay.

The reference cell line used in our study is the human T2 cell line, the HLA-A2 subtype of which is HLA-A*0201, TAP deficient and kept in our laboratory. It is used as HLA-A2 positive and HLA-A*0201 subtype positive control cell.

Genomic locus-specific primers for HLA-A2 and subtypes

According to the 12th International Histocompatibility Workshop, we established a nested PCR-SSP approach for discrimination of HLA-A2 alleles specificities designed on the ARMS (amplification refractory mutation system) principle and SSP binding, amplifying the complementary gene sequences principle (9, 10). The primer sequences and primer combinations are shown in Table 1 and Table 2 respectively. All the primers were synthesized by Invitrogen Biotechnology Co. Ltd., Shanghai. The primer combinations have been adapted to our amplification conditions based on previous description by Tonks et al. (9). In each reaction, a 256 bp region from exon 15 of the adenomatous polyposis (APC) gene was amplified simultanously as an internal control to certify the reaction functioned properly.

Preselection of HLA-A2-positive individuals

The first round PCR-SSP was performed using ARMS-PCR technology in 0.5 ml tube with thermocycler (TC-XP, Bioer Co.). A total of 25 μl reaction volume in each tube contained 100-300 ng template genomic DNA, 1.5 mmol/L $MgCl_2$, 2.0 μmol/L dNTPs, 0.4 μmol/L of each primer, including HLA-A2 specific primers (12ws & 33a) and internal control primers, 2.5 μl 10× Taq polymerase buffer, 1.25 U Taq polymerase (BioStar, Canada). At last 10 μl mineral oil overlaid. Thermal cycle conditions consisted of an initial denaturation step (96°C) for 5 minutes, followed by 5 cycles (96°C for 35 s, 70°C for 55 s, 72°C for 55 s), 21 cycles (96°C for 35 s, 65°C for 55 s, 72°C for 55 s) and 10 cycles (96°C for 35 s, 70°C for 55 s, 72°C for 55 s) of amplification, and a final extension (72°C) for 10 min. PCR products (5 μl) were electrophoresed in 1.5% agarose gels containing 0.5 μg/ml Goldview (Shanghai SBS Genetech Co.) after the addition of 1 μl of 6× loading buffer. The gels were run for 30 min at 15 V/cm in TBE buffer and visualized using a UV illuminator. Primers specific for HLA-A*0209 (12ws & 34a) and HLA-A*02015N (12ws & 35a) were also used in the first round of PCR.

To determine cell surface expression of HLA-A2 molecules, the isolated PBMCs were incubated with anti-HLA-A2 specific monoclonal antibodies (mAbs) BB7.2 (ATCC HB-82) for 30 min in 4°C. The cells were washed twice with PBS and incubated for 30 min on ice with FITC-labeled goat anti-mouse antibody. Fluorescence cytometic analysis was performed with FACSCalibur (BD

Table 1. Primers used in HLA-A2 subtyping

NO.	Position	Nucleotide sequence (5'-3')
12ws/101#	exon 2	GTGGATAGAGCAGGAGGGT
12ws/102#	exon 2	CCACTCCATGAGGTATTTCTT
12ws/103#	exon 2	CTCACTCCATGAGGTATTTCT A
12ws/104#	exon 2	CAGCTCAGACCACCAAGC A
12ws/105#	exon 2	TCCTCGTCCCCAGGCTCT
12ws/106#	exon 2	GAAGGCCCACTCACAGATTG
12ws/107#	exon 2	GGACGGGGAGACACGGAAA
12ws/108#	exon 2	GGACGGGGAGACACGGAAT
12ws/201#	exon 3	GAGCCACTCCACGCACTC
12ws/202#	exon 3	CTCCGCCTCATGGGCCGT
12ws/203#	exon 3	CACGTCGCAGCCATACATCA
12ws/204#	exon 3	GTGGCCCCTGGTACCCGT
12ws/205#	exon 3	ACCCCACGTCGCAGCCAT
12ws/206#	exon 3	CCGACCCCACGTCGCAGGCAC
12ws/207#	exon 4	CATGCTGCACATGGCAGGTT
12ws/208#	exon 3	CCTCCAGGTAGGCTCTAC
12ws/209#	exon 4	CTGGAAGGTTCCATCCCCTT
12ws/210#	exon 3	TACTGGTGGTACCCGCGC
12ws/211#	exon 3	CCTCCAGGTAGGCTCTCTG
*12ws/01#		ATGATGTTGACCTTTCCAGGG
*12ws/02#		ATTCTGTAACTTTTCATCAGTTGC

*Positive internal control primers

Table 2. Primer combinations and their specificity

Combination	Sense	Anti-sense	Size (bp)	Specificity
For the first round PCR				
12ws/33a	12ws/105#	12ws/204#	813	A*02
12ws/34a	12ws/104#	12ws/208#	971	A*02015N
12ws/35a	12ws/104#	12ws/210#	907	A*0209
For the second round PCR				
12ws/501	12ws/102#	12ws/209#	715	A*0201/A*0204/A*0207/A*0209/A*0211/A*02015N/A*0216/A*0217
12ws/502	12ws/102#	12ws/202#	694	A*0203
12ws/503	12ws/102#	12ws/203#	540	A*0204/A*0209/A*0217
12ws/504	12ws/102#	12ws/206#	549	A*0207/A*02015N
12ws/505	12ws/103#	12ws/208#	715	A*0206/A*0210/A*0214
12ws/506	12ws/106#	12ws/208#	522	A*0211
12ws/507	12ws/101#	12ws/201#	595	A*0216
12ws/508	12ws/103#	12ws/205#	549	A*0205/A*0206/A*0208/A*0214
12ws/509	12ws/102#	12ws/205#	547	A*0201/A*0202/A*0203/A*0204/A*0209/A*0211/A*0212/A*0213/A*0216
12ws/510	12ws/108#	12ws/210#	408	A*0208
12ws/511	12ws/107#	12ws/210#	409	A*0202/A*0205/A*0214
12ws/512	12ws/102#	12ws/211#	705	A*0212/A*0213

Co.).

HLA-A2 subtyping

The PCR product of the first round amplification with primer combination 12ws & 33a was diluted 1:50 with double distilled water, and 1.0 μl of the diluted DNA was used as template in the next PCR amplification for HLA-A2 subtyping. The reaction volume is 10 μl, which includes 0.4 μmol/L of each subtype-specific primer and 0.1 μmol/L of the internal positive control primer, in addition to the rest components used in the first round of PCR. The nested PCR cycling was preformed as follows: 96°C for 1 min, 10 cycles of (96°C for 20 s, 65.7°C for 45 s, 72°C for 25 s), 5 cycles of (96°C for 20 s, 59.5°C for 45 s, 72°C for 25 s), 5 cycles of (96°C for 20 s, 55°C for 45 s, 72°C for 25 s), and 96°C for 10 min. The products of PCR were determined with the same step described above.

DNA sequencing for PCR product

The PCR products of first round amplification with HLA-A2 specific primers combination (12ws & 33a) were electrophoresed in 1.5% agarose gels. The 813 bp size band, which is an HLA-A2 DNA fragment covering a region from exon 2 to exon 4, was purified with Biospin Gel Extraction Kit (Cat. No. BSC02M1, Bioer Technology Co. Ltd.). The purified amplification products were sequenced by Invitrogen Biotechnology Co. Ltd., Shanghai. The subtype could be determined by sequenced results blasted with gene bank of HLA-A2 (6).

Statistical analysis

HLA-A2 phenotype frequencies in the tested samples were estimated by direct counting. The allele frequencies were estimated by the square root method. Hardy-Weinberg equilibrium was calculated by standard methods and tested by chi-square goodness of fit.

Results

The first round of PCR-SSP typing for HLA-A2 prescreening

The prescreening of HLA-A2 samples was preformed by using the PCR-based typing with A2-group specific primer combination (12ws & 33a). Seventy-eight HLA-A2 positive samples were screened from 154 random samples. The results of the first round PCR-SSP for some samples were showed in Figure 1. The PCR-SSP typing result was verified by FCM analysis with the anti-HLA-A2 mAb (BB7.2). The

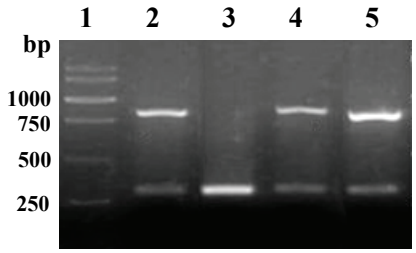

Figure 1. A typing result of the first round PCR-SSP with HLA-A2 group specific primer combination (12ws & 33a). The products of PCR were determined with 1.5% argrose gel electrophoresis. The HLA-A2 specific band is 813 bp in size and the positive internal control is 256 bp. Lane 1, loaded with DNA marker; Lane 2, T2 cell line for HLA-A2 positive control; Lane 3, a HLA-A2 neg ative sample; Lanes 4 and 5, two HLA-A2 positive samples.

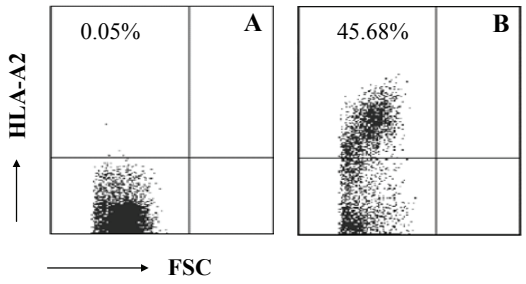

Figure 2. FCM analysis reveals the expression of HLA-A2 molecule on the surface of PBMCs from tested samples. PBMCs were incubated with HLA-A2 specific mAbs BB7.2 and FITC-labeled goat anti-mouse antibody, then visualized by FCM analysis. (A) shows no HLA-A2 molecule expressed on the PBMCs of a sample which was determined as HLA-A2 negative in the first round PCR-SSP typing. Part B is the FCM analysis result of a sample of HLA-A2 positive, suggesting significant HLA-A2 molecule can be detected by HLA-A2 specific mAbs BB7.2.

PBMC samples of HLA-A2 positive cases were observed to be bound by the BB7.2 specifically. As shown in Figure 2, the PCR-SSP typed HLA-A2 positve PBMCs can be bound by the BB7.2 by FCM analysis.

The second round PCR-SSP for HLA-A2 subtyping

The HLA-A2 subtyping was preformed by the second round of PCR-SSP using a panel of 12 combinations of 14 allelic SSP listed in Table 2, with the amplification product of the first round PCR-SSP as templates. The sample of T2 cell line was subtyped to be HLA-A*0201 with this nested PCR-SSP DNA subtyping method. This result is coherent with the description of T2 from ATCC. Figure 3 shows the result of the second round PCR-SSP for a sample identified as HLA-A*0201. We found HLA-A*0201, HLA-A*0207, HLA-A*0206, HLA-A*0203 and HLA-A*0210 in the studied blood samples.

Reliability assessment of HLA-A2 subtyping

The HLA-A2 subtypes were determined by the method of PCR-SSP with high resolution, whose reliability was assessed by the standard cell line and direct sequencing. The standard control cell line T2 carrying HLA-A*0201 was verified by our subtyping method. The amplification products of first round PCR-SSP for the subtypes detected in this study were sequenced. The sequenced results were coherent with the HLA-A2 subtypes typed by the above nested PCR-SSP subtyping. Furthermore, the complete encoding region of the subtypes were cloned and confirmed by sequencing, which were expected for further allelic function study.

Distribution of HLA-A2 alleles among Wuhan population

Of the 154 individuals included in our study, 78 (50.7%) were typed to be HLA-A2 positive by the PCR-SSP and to express HLA-A2 molecules on cell surface by the FCM analysis. A total of five alleles of A2 subtypes were detected among the 78 HLA-A2 positive individuals. These alleles were A*0201 (15.5%), A*0207 (5.8%), A*0206 (4.7%), A*0203 (2.6%) and A*0210 (0.7%). With this approach, only one sample of HLA-A2 positive was undetermined for its HLA-A2 subtype. No other A2 subtype alleles, such as A*0202 or A*0204, were found in the study. Distribution of the HLA-A2 alleles was in accordance with Hardy-Weinberg equilibrium ($p > 0.5$), and hence these allele frequencies may represent the distribution pattern of the HLA-A2 alleles among Wuhan population. The profile of HLA-A2 alleles in Wuhan population is shown in Table 3.

Discussion

Serological HLA-A2 specificity is encoded by at least 75 different alleles, and many studies have shown that there are significant ethnic differences in the distribution of HLA-A2 alleles (4, 14, 15). HLA-A*0201 has been found in all populations studied, with a stronger predominance in Western Caucasian populations than Asian populations. In comparison, the majority of other HLA-A2 alleles appear to be specific to particular ethnic groups. A*0202 and A*0214 are specifically found in blacks and A*0203, A*0206, A*0207 and A*0210 are characteristic of Asian populations (14-16).

Because HLA-A2 is one of the dominating class I allele

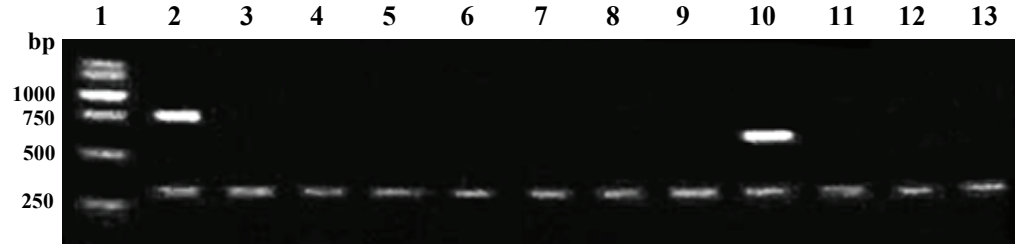

Figure 3. HLA-A2 subtyping result of the second round PCR-SSP with a panel of 12 combinations of 14 allelic SSP. Lane 1, DNA marker. Lanes 2 to 13, PCR-SSP products of an HLA-A2 positive sample amplified with SSP combinations of 12ws/501 to 12ws/512 corresponding to the order listed in Table 2. An internal control gives a 256 bp band in size for every amplification, indicating the reaction works properly.

Table 3. Distribution of HLA-A2 alleles in a Wuhan population (n = 154)[1]

Allele	n[2]	PF (%)[3]	AF (%)[4]
A*0201	44	28.6	15.5
A*0203	8	5.19	2.6
A*0206	14	9.09	4.7
A*0207	18	11.4	5.8
A*0210	2	1.3	0.7
A*02UD[5]	1	0.6	0.3

[1]A total of 154 individuals were analyzed, the HLA-A2 positive individuals detected by the PCR-SSP and the FCM were further tested for A2 alleles.
[2]Among the 78 $A2^+$ individuals, 87 A2 alleles were detected by direct counting.
[3]Phenotype frequency.
[4]Allele frequency.
[5]A2 allele of undetermined type with this approach.

groups, various strategies were developed to specifically subtype HLA-A2 for population genetics and tissue transplantation (11). The number of newly discovered HLA-A2 alleles is ever-increasing with the population studies progressing so that eventually only direct sequencing may suffice for all their discrimination. Substantial increases in time and cost are incurred if the typing approach is utilized to discriminate those rare subtypes. The method described here can determine 17 subtypes, including the frequent HLA-A2 alleles in Chinese (> 99% allele frequency) reported by previous studies (1, 12, 13). It is rapid, inexpensive and highly adaptable to clinical and laboratory studies for HLA-A2 subtyping in Chinese. The two round PCR reactions can exclude the intereference of other non-HLA-A2 class I alleles and enhance the specificity of the reactions. The quality or quantity of Taq enzyme and DNA templates can influence the final subtyping results. We have found the suitable conditions as the above depicted in our laboratory by reduplicated experiment. It can be more easily introduced to be a routine detecting method compared to direct sequencing of high demanding instruments and serological reactions of unavailable subtype specific antibodies. The entire molecular procedure takes about 8 h, including 5 h for the first step and 3 h for the second step. The subtyping results of this nested PCR-SSP confirmed by DNA sequencing demonstrate the reliability of the approach. The described strategy provides a feasible method for quick and reliable HLA-A2 subtyping in Chinese population.

In the present study of 78 HLA-A2 positive individuals of Wuhan population, substantial heterogeneity of HLA-A2 alleles was observed. Five different HLA-A2 alleles were detected, i.e., HLA-A*0201, A*0203, A*0206, A*0207 and A*0210. The four most common HLA-A2 alleles were A*0201 (15.5%), A*0207 (5.8%), and A*0206 (4.7%) with a presence of A*0203 at a low frequency of 2.6%. These four alleles were also detected in other Chinese populations with overall frequencies similar to those of the present study. However, the frequencies of A*0203, A*0206, A*0207 were much lower, or even not detected in Caucasian (14).

The clustering of certain HLA-A2 alleles in different ethnic supports the view that these alleles have been mutated and been selected in response to locally prevalent pathogens (3). The ubiquitous presence of A*0201 allele suggests that it is particularly important in terms of protective immunity. In this respect, the A*0201 gene product has been shown to present peptides from several ubiquitous intracellular pathogens, including influenza virus, Epstein-Barr virus, hepatitis B virus and malaria (3-5). In a study of mutant HLA-A2 molecules, substitution of nonpolar phenylalanine by polar tyrosine at residue 9 corresponding to A*0206 had a dramatic negative effect on binding of the HIV peptide and the influenza A matrix peptide. Similarly, substitution of polar tyrosine by less polar cysterine at residue 99 corresponding to A*0207 was shown to affect presentation of three different viral peptides (17). Epstein-Barr virus $EBNA3_{596\text{-}604}$ peptide is the only epitope which is known to be HLA-A*0203 restrictive (18). These HLA-A2 variants can elicit strong allogeneic responses and this has implications for matching donor and recipient for transplantation (19). A recent study in Japanese has shown that HLA-A2 allele disparity has an important effect on acute graft-versus-host disease and survival rates in unrelated hematopoietc stem cells transplantation (8, 19). In Chinese, mismatch of HLA-A2 alleles would be also frequent for the high frequency of HLA-A2 (> 50%) and the substantial heterogeneity of alleles. These differences in antigen binding and presentation among HLA-A2 alleles may have relevance for matching unrelated donor-recipient pairs at the sequence level for marrow transplantation and adoptive immune therapy studies (20).

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