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Short Communication

Specific genotyping of human leukocyte antigen-A*01 by polymerase chain reaction using allele group-specific primers

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Abstract

We established a specific genotyping assay for HLA-A*01, which is one of the most frequently found HLA-A alleles in the Caucasian population. This assay uses the polymerase chain reaction (PCR) with allele group-specific primers (ASP). HLA-A*01 group-specific primers were designed for exon 3 of the HLA-A gene, based on the recent HLA-sequence alignment. Both sense and anti-sense primers were designed with completely matched sequences to each specific HLA-A*01 allele, but mismatched by at least 1 nucleotide to all other known class I HLA alleles. By the use of these primers and stringent PCR conditions, we successfully genotyped the HLA-A*01 group alleles and achieved greater accuracy than previous methods.

Key words: HLA-A1, PCR, allele group-specific primers.

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The human leukocyte antigen (HLA) exhibits the highest degree of polymorphism in the human genome (Tokunaga *et al*., 1997; Prasad and Yang, 1996), and more than 950 HLA class I alleles have been reported to date (www.anthonynolan.org.uk/HIG/lists/class1list.html). Until recently, serological typing has been the primary technique used for HLA class I typing (Mittal *et al*., 1968). However, with the advent of PCR technologies, DNA-based HLA genotyping has developed (Sadler *et al*., 1994; Browning *et al*., 1993) during the last few years and has gradually replaced serological methods. Most commonly, DNA-based genotyping for the HLA-locus is performed by PCR with combinations of allele-specific primers (ASP) (Newton *et al*., 1989) designed from one of the hypervariable regions located in the second and third exons which encode the extracellular domains (Trowsdale *et al*., 1991). PCR-ASP methodology is based on the principle that completely matched oligonucleotide primers are more efficiently used in amplifying a target DNA sequence than mismatched oligonucleotide primers (Newton *et al*., 1989), and a mismatch at the 3' end of the primer to non-target sequence inhibits non-specific amplification. However, it is difficult to apply this technique for HLA group-specific genotyping, since HLA genes have a complex structure and have sequence homology among the different HLA class I loci, including the less polymorphic HLA-E, F, G genes and several pseudogenes (Trowsdale *et al*., 1991). Recently, the search for new class I alleles has advanced rapidly, and detection of new HLA alleles (Bunce *et al*., 2000; Guttridge and Darke, 2001) may render PCR-ASP techniques obsolete. For this reason, group-specific primers are often designed with a mismatch at different positions from the 3' end, and this reduces the amplification efficiency and specificity. In addition, many of the HLA class I group-specific primers are now designed to match the major alleles of each HLA group, but some less common alleles are mismatched to these primers. As a result, false-positive and false-negative amplification occur by the combination of these ASPs. Recently, HLA typing has contributed not only to the disease association studies, but also to the fields of legal medicine (Primorac *et al*., 1996) and anthropology (Ivanova *et al*., 2001; Arnaiz-Villena *et al*., 1999). Therefore, it is important to design primers which are completely matched to the target HLA loci, to provide accurate HLA group-specific genotyping.

In the present study, we established a new method of HLA-A*01 genotyping, which is one of the most frequent HLA-A allele groups in the Caucasian population. We designed the HLA-A*01 group-specific primer and PCR conditions based on the most up-to-date HLA-sequence alignment (http://www.ebi.ca.uk/imgt/hla/align.html). Both sense and antisense primers were designed in exon 3

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of the HLA-A gene. The PCR product size is 101 bp. Although the sense primer is matched to all 11 HLA-A*01 alleles, this primer is also matched to some other HLA-A alleles (HLA-A*11 group, A*36 group, HLA-A*0244, HLA-A*24022 and HLA-A*6812) (Figure 1a). Other HLA-A alleles are mismatched by at least one nucleotide to the sense primer sequence, and HLA-B and C locus alleles are mismatched by at least two nucleotides. The antisense primer is only matched to all 11 HLA-A*01 alleles. Other HLA-A alleles are mismatched by at least one nucleotide at the 3' end or by two nucleotides in the centre of the primer, and HLA-B and Cw locus alleles are mismatched by at least 2 nucleotides (Figure 1b). With the exception of HLA-A*11 (2-nucleotide mismatch) and HLA-A*24022 (1 nucleotide mismatch at 3' end), these primers have at least 3 nucleotides mismatched to other HLA-A alleles. All HLA-B and HLA-Cw alleles have at least 4 mismatched nucleotides, and other class I HLA locus (HLA-E, F, G) alleles have more than 10 nucleotides mismatched to these primers. Finally, the sequences of these primers were completely matched to only the 11 HLA-A*01 alleles. In order to verify whether all DNA is successfully amplified or not, internal control primers which amplified a 394 bp fragment of the Coxsackie-adenovirus receptor gene (Bowles *et al*., 1999) (exon 2) were added in each reaction mixture. The sequence of the control sense primer is 5'-CTGGGCAT CTCTTGAGTTTGGA-3', and the anti-sense primer is 5'-ACTGGCAAGGTGATGGACACAT-3'. Optimized PCR conditions for HLA-A*01 typing are as follows: the PCR reaction mixture in a final volume of 20 μL consisted of 100 ng genomic DNA, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.1 μM of each HLA-A*01 primer and 1.0 μM of each control primer, and 0.5 U DNA Taq Polymerase (Hotstar Taq®, Qiagen Inc, Mississauga, Ontario). PCR amplification was performed in a PCR EXPRESS Thermal Cycler (Thermo Hybrid, Ashford, Middlesex, UK). PCR conditions for cycling were optimized using the touchdown method (Hecker and Roux, 1996) as follows: initial denaturation step at 95 °C for 15 min, followed by 5 cycles of 94 °C for 30 s (denaturation), 70 °C for 30 s (annealing), and 72 °C for 45 s (extension), followed by 10 cycles with a decreased annealing temperature at 69 °C and 20 cycles at 68 °C, and a final extension step of 10 min at 72 °C.

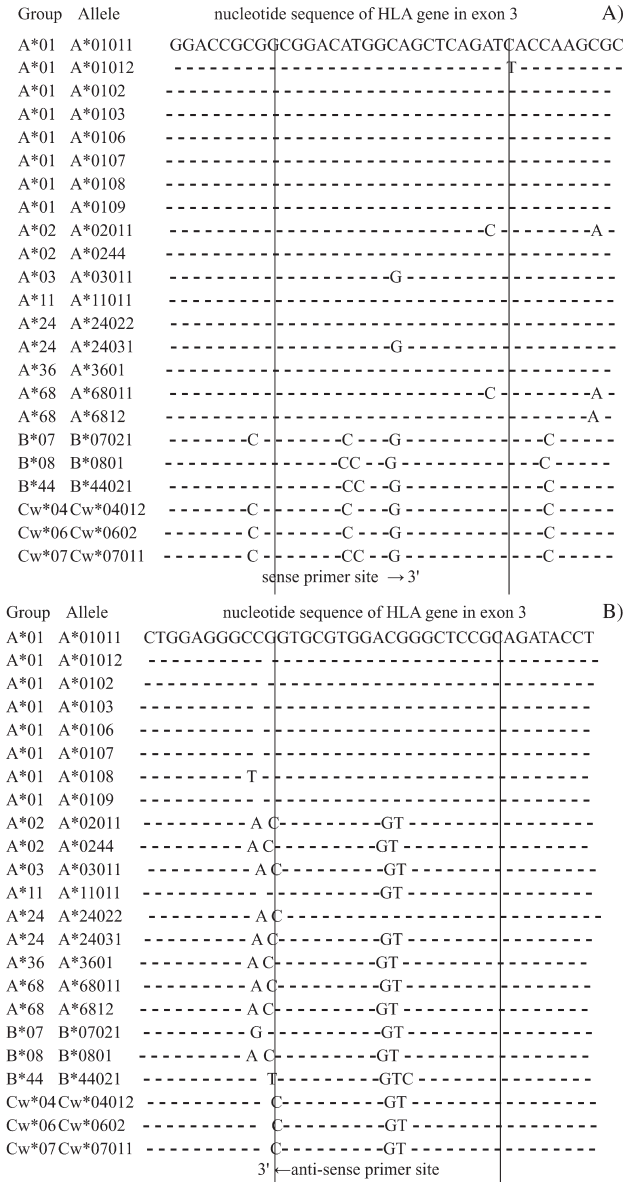

Figure 1 - Comparison of nucleotide sequence alignment showing exon 3 of class I HLA genes. All HLA-A*01 alleles and other HLA-A allele groups which have similar sequence to HLA-A*01 are shown. Other representative class I HLA alleles are also shown. A dash (-) indicates identity between the sequences. The nucleotide sequence surrounding sense primer site. The nucleotide sequence surrounding anti-sense primer site.

Figure 2 represents the amplified PCR fragment of HLA-A*01 and the internal control. The 101 bp band in lane 6 is specific for HLA-A*01 (Figure 2a and 2b). Changes in reagent and high DNA concentration or changes of annealing temperature may cause the ambiguous result shown in Figure 2b. In this case, a 2 °C lower annealing temperature causes false-positive bands such as in lane 5. This sample was actually HLA-A*11. We successfully genotyped the HLA-A*01 positive sample by using stringent PCR conditions and distinguished it from the other alleles even when the mismatch was not at the 3' end of the primers. After optimization of the PCR conditions, we performed HLA-A*01 genotyping using 200 Caucasian DNA samples. We could evaluate HLA-A*01 positive bands in this large number of samples without ambiguous amplification (Figure 3). Positive samples were selected for direct sequencing, which confirmed the genotypes as HLA-A*01. We calculated the allele frequency of HLA-A*01 in the 200 genotyped samples by assuming Hardy-Weinberg equilibrium. The estimated allele frequency of HLA-A*01 was 0.15, which was well matched to the reported Caucasian HLA-A*01 allele frequency. Thus, we consider that by the use of these primers and stringent PCR conditions, we suc-

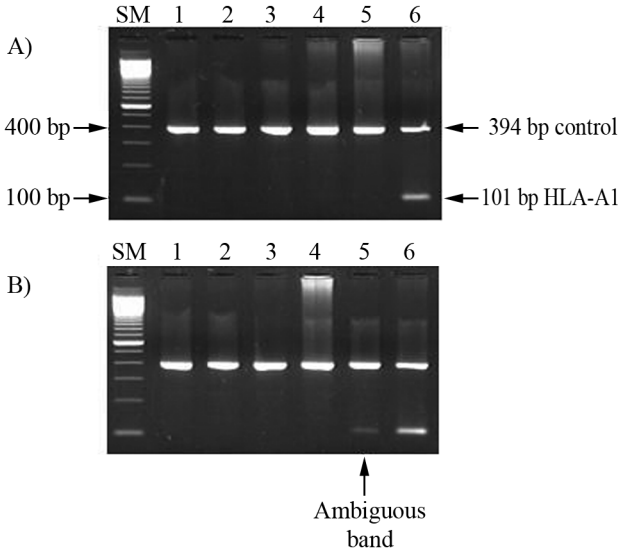

Figure 2 - Band pattern of HLA-A*01 genotyping. a. HLA-A*01 genotyping result based on the optimized PCR conditions. The visible band at 101 bp in lane 6 is derived from the HLA-A*01 allele. A control band at 394 bp can be seen in each lane, indicating that genomic DNA from each sample was successfully amplified. b. HLA-A*01 genotyping result using 2 °C lower annealing temperature. The visible band at 101 bp in lane 6 is derived from the HLA-A*01 allele. Under these conditions, an ambiguous band is present in lane 5. This individual was HLA-A*11, which has sequence similarity to HLA-A*01 at the primer sites.

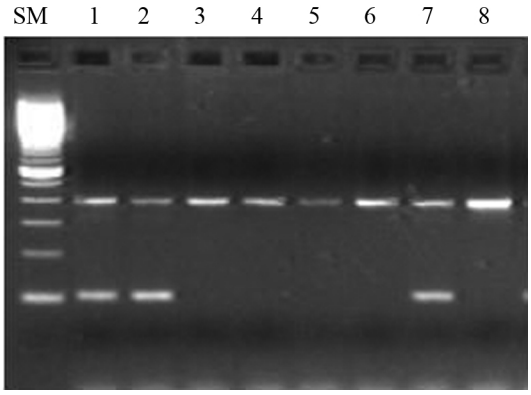

Figure 3 - Representative result of HLA-A*01 genotyping. Lane 1, 2 and 7 show a positive band, indicative of the HLA-A*01 allele.

cessfully genotyped the HLA-A*01 group alleles and distinguished them from 952 HLA class I alleles.

PCR-ASP is one of the common methods for group-specific HLA typing, but it sometimes leads to typing errors (Schaffer and Olerup, 2001). In fact, we previously used the published group-specific primers for class I HLA genotyping, and occasionally found mistyping by direct sequencing. One of the reasons for mistyping is sub-optimal design of ASPs. For example, in the case of HLA-A*01 group-specific amplification, the primer sequences of HLA-A*01 are matched to the high-frequency alleles such as HLA-A*010101 and HLA-A*0102, whereas these are not completely complementary to HLA-A*0103, HLA-A*0106 or HLA-A0107. Moreover, other HLA-A group alleles (*e.g.*, HLA-A*3603, HLA-A*8001) are matched to this sequence and amplified by these primers. In this study, however, we also found that minor changes of primer and enzyme concentration, or annealing temperature, could cause the typing error shown in Figure 2b even with the use of highly specific primers. Therefore, we emphasize that strictly controlled PCR conditions as well as the use of well-designed primers are required to achieve accurate HLA group-specific genotyping. On the other hand, HLA-A*01 is one of the most frequent serological types in the Caucasoid population, with an allele frequency of 0.13-0.18. From these data, it results that almost 25-34% of Caucasian people have HLA-A*01. Thus, precise HLA-A*01 typing would provide important information for disease and population studies.

In summary, we established a new and accurate genotyping method for HLA-A*01 by using PCR-ASP. The primers successfully amplified all 11 HLA-A*01 alleles and distinguished them from all other HLA class I alleles. Primer design and allele identification were according to the newly-aligned HLA sequence database. Thoroughly optimized, appropriate PCR conditions were also used to prevent both false-positive and false-negative results. Thus, we overcame the drawback of previous PCR-ASP methods for HLA typing as mentioned above, and provided a reliable HLA-A*01 genotyping method.

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