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Detection of Single Nucleotide Polymorphism in Human IL-4 Receptor by PCR Amplification of Specific Alleles

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A key aspect of genomic research in the "post-genome era" is to associate sequence variations with heritable phenotypes. The most common variations in the human genome are single nucleotide polymorphisms (SNPs) that occur approximately once in every 500 to 1,000 bases. Although analyzing the phenotypic outcome of these SNPs is crucial to facilitate large-scale association studies of genetic diseases, detection of SNPs from an extended number of human DNA samples is often difficult, labor-intensive and time-consuming. Recent development in SNP detection methods using DNA microarrays and mass spectrophotometry has allowed automated high throughput analyses, but such equipments are not accessible to many scientists. In this study, we demonstrate that a simple PCR-based method using primers with a mismatched base at the 3'-end provides a fast and easy tool to identify known SNPs from human genomic DNA in a regular molecular biology laboratory. Results from this PCR amplification of specific alleles (PASA) analysis efficiently and accurately typed the Q576R polymorphism of human IL4 receptor from the genomic DNAs of 29 Koreans, including 9 samples whose genotype could not be discerned by the conventional PCR-SSCP (single strand conformation polymorphism) method. Given the increasing attention to disease-associated polymorphisms in genomic research, this alternative technique will be very useful to identify SNPs in large-scale population studies.

Analysis of genetic variation in the human genome can shed light on the problem of the molecular basis of many complex disorders. The majority of the known cause of human genetic disorders is associated with single nucleotide polymorphism (SNP), the most common type of sequence variation estimated to occur every 500 to 1,000 bp in the genome (Cooper et al, 1985; Harding et al., 1997). Due to this high frequency, SNPs are particularly important tool for the mapping of susceptibility genes in complex trait diseases such as cancer, diabetes, obesity, mental illness and autoimmune disorders, which require high-density genetic markers (Kruglyak, 1997). Indeed one major goal of the Human Genome Project had been assigned for identification of these genetic variants and their application in genome-wide disease association studies (Collins et al., 1998).

Unfortunately, detection of a single base variation from a large number of human DNA samples often faces one or more technical difficulties. RFLP (restriction fragment length polymorphism) and SSCP (single strand conformation polymorphism) have been two major methods of identifying SNPs from PCR-amplified genomic fragments. The PCR-RFLP method, when applicable, provides a simple and relatively clear diagnosis of SNPs, however only a small subset of SNPs creates changes in the pre-existing restriction sites. The PCR-SSCP analysis can be used with less limitation; yet the changes in the SSCP band pattern produced by some SNPs are not easily discernable, let alone the labor intensive nature of the method when applied to a large number of samples. One way to circumvent these problems is to take advantage of the fact that PCR primers containing a mismatch at the most 3'-end cause a significant decrease in the amplification efficiency. Variations of this technique have been adopted in the typing of HLA subtypes (Zetterquist and Olerup, 1995) and point mutations in phenylketonuria (Sommer et al., 1989), using primers designed to represent a particular type of SNP variations at the 3-end. However, this simple and convenient method is not widely used in the field of SNP analysis.

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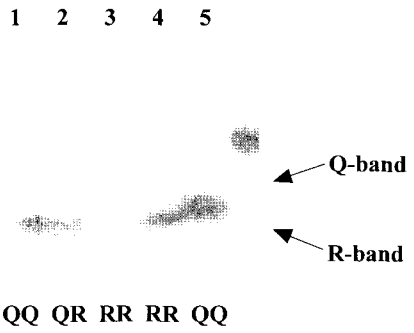

Fig. 1. Typical band patterns from the PCR-SSCP genotyping of the Q576R polymorphism in the human IL-4R gene. Genomic DNAs of Korean SLE patients were analyzed for the known Q576R polymorphism in the IL-4R gene by PCR-SSCP method as described in Hwang et al (2000). The upper band represents the PCR product containing Q-type allele, and the lower band represents the DNA containing R-type allele.

In this study we applied the technique of PCR amplification of specific alleles (PASA) to the typing of a known SNP in human IL4 receptor, which causes the Q576R substitution. The role of IL4 as a suppressor cytokine suggests that genetic variations in the IL4R locus may be linked with immune disorders. Previously the Q576R polymorphism was shown to be associated with atopic disease (Hershey et al., 1997), and its possible association has also been indicated in the study of autoimmune disorders (Hwang et al., 2000). In these analyses a PCR-SSCP method was used to type the A to G substitution at nucleotide 1902 that changes the amino acid residue 576 from glutamine (Q) to arginine (R). However, it was often difficult to distinguish the conformational difference between the two genotypes on the SSCP gel. Here we demonstrate that a parallel PASA analysis using two independent primers each containing SNP variation at the 3'-end is a convenient and accurate alternative. With proper optimization in the PCR condition for each target sequence, the PASA method provides a simple and generally applicable tool to detect known SNPs in large-scale population screening analyses.

Materials And Methods

Subjects

Genomic DNAs from 29 Korean patients with systemic lupus erythematosus (SLE) that had been typed for the Q576R variation in human IL4 receptor locus by PCR-SSCP method (Hwang et al., 2000) were recruited in this study. Specifically, DNAs from 20 patients typed to *be homozygote (QQ and RR) or heterozygote (QR)*, according to the presence of upper or lower SSCP bands (Fig. 1), were tested by the PASA analysis. Also, DNA samples from 9 patients whose SSCP result produced ambiguous band patterns were included in the typing by the PASA method.

Conditions for PASA analysis

Primers to selectively amplify the Q576 (adenine at the nucleotide 1902) and R576 (guanine at the nucleotide 1902) alleles were designed as diagramed in Fig. 2. The common upstream primer (5'-AGGCAACCCTGC-TTACCGCA-3') was used in pair with either the Q576 allele specific primer (5'-CACCGCATGTACAAACTCCT-3') or with the R576 allele specific primer (5'-CACCGC-ATGTACAAACTCCC-3'). PCR amplification was performed using 50 ng of template genomic DNAs from patients. PCR reaction contained 1.5 unit of *Taq* polymerase (Takara, Japan), 10 μM each of upstream and downstream primers, and 2.5 mM of each dNTP. Three different concentrations of $MgCl_2$ in the reaction buffer, e.g. 1.5 mM, 1.25 mM and 1.0 mM, were used. The reaction was preheated at 94℃ for 5 min, then run through 35 cycle of 30 sec 94℃-1 min 60℃-30 sec 72℃. The amplified product was electrophoresed on 1.5% agarose gel containing ethidium bromide.

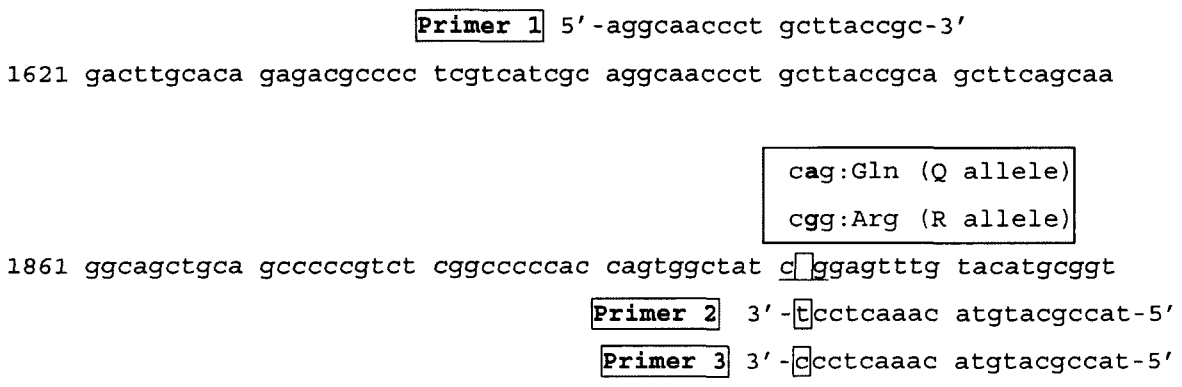

Fig. 2. Structure of PCR primers specific to each allele of IL-4R polymorphism. Primers for PASA analysis were designed as the common upstream primer (primer 1), and two downstream primers each designed to complement A (primer 2) or G (primer 3) nucleotide, respectively, at the 3'-end. Numbers on the left represents the nucleotide position as shown in the GenBank entry of human IL-4R (ID number X52425). Site of the Q576R polymorphism at position 1902 was designated by boxed letters.

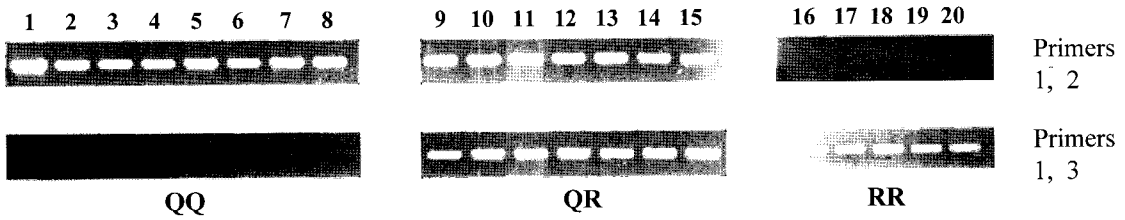

Fig. 3. Specific amplification of Q and R alleles of human IL-4R genomic region. Genomic DNAs from individuals typed as QQ by the PCR-SSCP method were amplified only with primer 1 and primer 2 (lanes 1 to 8), while DNAs from RR individuals were amplified only with primer 1 and primer 3 (lanes 16-20). Genomic DNAs that are typed to be heterozygotes (lanes 9-15) were amplified with both primer combinations.

DNA sequencing of the genomic region containing Q576R polymorphism

To confirm the typed results from PASA, the region containing Q576R polymorphism was cloned by PCR amplification and sequenced. For the accuracy of DNA sequencing, primers were designed to contain the region of SNP in the middle of the PCR product (5'-CCGAAATGTCCTCCAGCATG-3' for upstream primer and 5'-CCAGTCCAAAGGTGAACAAGGGG-3' for downstream primer). PCR amplification was done on the 50 ng of genomic DNA using the same amount of *Taq* polymerase, primers and dNTPs as described above. The reaction was preheated at 94℃ for 3 min, then run through 35 cycle of 1 min 94℃-1 min 58℃-1 min 72℃. The integrity of amplified products was tested on an agarose gel, then 1 μl of this was TA-cloned into the pGEM-T-easy plasmid, and analyzed by automated dideoxy sequencing.

Results And Discussions

Typing of the Q576R polymorphism in the human IL-4R by PCR analysis using primers containing the altered nucleotide at the 3'-end produced results that are in good accordance with the previous SSCP-based genotyping (Fig. 3). PCR amplification using primer 1 and primer 2 produced the expected 271 bp product in all 8 samples typed to be QQ by the PCR-SSCP method, while the combination of primer 1 and primer 3 failed to amplify the corresponding allele. Vice versa, all 5 samples that were typed to be RR by the SSCP method were amplified only when the combination of primer 1 and primer 3 was applied. On the other hand, genomic DNAs from the 7 patients typed as QR heterozygote were amplified with both combinations of primers. Taken together, it was proven that the presence of a single nucleotide mismatch at the 3'-end of primer 2 and primer 3 accurately discriminated the Q and R allele of the *IL-4R* genomic region.

Table 1. Confirmation of PASA typing by DNA sequencing

Specimen No.	PASA typing	DNA sequencing	
		No. of analyzed clones	Detected allele types
1	QQ	5	5 Q 0 R
2	QQ	5	5 Q 0 R
3	QR	4	2 Q 2 R
4	QR	4	2 Q 2 R
5	QR	5	3 Q 2 R
6	QQ	4	4 Q 0 R
7	QQ	5	5 Q 0 R
8	QR	5	3 Q 2 R
9	QQ	5	5 Q 0 R

One major drawback in the SSCP-based genotyping of the Q576R polymorphism was the fact that approximately 30% of the tested genomic DNAs produced ambiguous band patterns. This is mainly due to the relatively weak intensity of the R-type band and the closeness of the two bands (Fig. 1). However, the possibility of additional SNP within the PCR product affecting the SSCP conformation cannot be ruled out. We tested whether the PASA analysis could identify the correct genotype of 9 individuals whose SSCP analysis failed to produce clear band patterns. All 9 genomic DNA samples were unambiguously typed to be either QQ or QR by parallel PCR amplifications, using the combination of primer 1 and primer 2 (which detects the Q allele) and primer 1 and primer 3 (which detects the R allele) (Table 1, left column). None of these DNAs were RR homozygote, as expected from the fact that the RR genotype is the least likely to be mistyped by SSCP analysis. Results from the PASA typing were confirmed by DNA sequencing of 4-5 randomly chosen clones containing PCR amplified genomic region of each patients' IL-4R loci. All clones from individuals typed as QQ homozygotes had A nucleotide at position 1902, while clones from heterozygotes contained either A or G nucleotide at corresponding position (Table 1, right column).

It is known that the activity of *Taq* polymerase is affected by the concentration of $MgCl_2$ in the PCR reaction buffer, and modulation of the final $MgCl_2$ concentration is often applied to abolish non-specific PCR amplification. In this study optimal $MgCl_2$ concentration was critical to achieve the differential amplification of specific IL-4R alleles (Fig. 4). The presence of 3'-end mismatch did not affect PCR amplification in the buffer containing 1.5 mM $MgCl_2$, as demonstrated by the amplification of both Q and R allele with either primer 2 or primer 3. However, primer 2 and primer 3 recognized only the template DNA with perfect match in 1.25 mM $MgCl_2$. In the buffer containing 1 mM $MgCl_2$, both primers failed to amplify even the cognate tem-

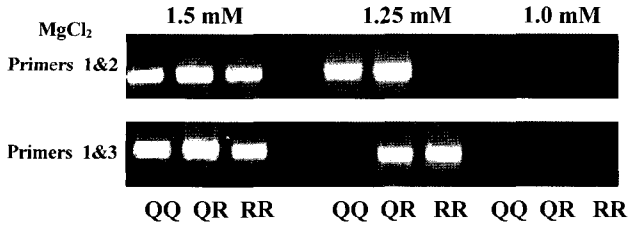

Fig. 4. Effect of the $MgCl_2$ concentration in the allele-specific PCR amplification. Both Q and R alleles were amplified with either combination of primers (e.g. primer 1 and primer 2 vs. primer 1 and primer 3) in the PCR reaction buffer containing 1.5 mM $MgCl_2$ (lanes 1 to 3), while each allele was amplified only with the cognate downstream primers when the $MgCl_2$ concentration was lowered to 1.25 mM (lanes 4 to 6). In a buffer containing 1 mM $MgCl_2$, even the cognate primers failed to amplify neither Q nor R allele of the IL4R genomic region (lanes 7 to 9).

plate. However, the optimal condition for allele-specific amplification may differ from one genomic region to the other, and requires to be adjusted for each target sequence.

Taken together, the PASA method tested in this study provided a convenient genotyping of DNA samples using a simple PCR-based analysis. With the increasing attention to the discovery and characterization of SNPs in the human genome, this technique will serve as a simple and generally applicable tool to detect disease-related polymorphisms in high throughput screening analyses.

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