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業績報告書シリーズ

PCR-SSCP法および制限酵素切断法によるHLA-DQA1遺伝子タイピングのための迅速・簡便な方法§

Simple, Rapid HLA-DQA1 Genotyping Using the Polymerase Chain Reaction and Analysis by Single-strand Conformation Polymorphism and Restriction-enzyme Cleavage

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要　約

HLA-DQA1遺伝子座の対立遺伝子を解析するための迅速で簡便な方法を開発した。ポリメラーゼチェイン反応(PCR)によりHLA-DQA1遺伝子座の多型性に富む第2エクソンを増幅した。増幅したDNAは一本鎖高次構造多型分析法(SSCP法)および制限酵素切断法を用いて解析を行った。この方法を用いることにより，HLA-DQA1の8種の対立遺伝子のすべてを区別することが可能であった。この方法はHLA-DQA1対立遺伝子の迅速な遺伝子タイピングとして用いられるばかりでなく，フラグメント中の各部位の点突然変異の検出およびHLA-DQA1の新しい遺伝子タイプの検出にも有用であると判断した。

§本業績報告書は研究計画書RP 4-90に基づく。本報告に基づく論文はElectrophoresis 13:877－9, 1992に掲載された。本報告にはこの要約以外に訳文はない。承認1992年12月16日。印刷1993年6月。

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Simple, Rapid HLA-DQA1 Genotyping Using the Polymerase Chain Reaction and Analysis by Single-strand Conformation Polymorphism and Restriction-enzyme Cleavage§

Tomonori Hayashi, Toshio Seyama, Takashi Ito, Yoichiro Kusunoki, Yuko Hirai, Nori Nakamura, Mitoshi Akiyama

Summary

A simple and rapid method for identifying alleles at the human-leucocyte-antigen-DQA1 locus (HLA-DQA1 locus) is described. The polymorphic second exon of the HLA-DQA1 locus was amplified by the polymerase-chain-reaction method. The amplified DNA was then analyzed by the single-strand-conformation-polymorphism/restriction-enzyme-cleavage assay. Using this method, the eight known DQA1 alleles could be distinguished from each other. In this paper, this method is suggested for quick genotyping of DQA1 alleles, but this method should also be useful for detecting point mutations at various positions in a fragment as well as new HLA-DQA1 genotypes.

Introduction

Researchers elsewhere have postulated that the survivors of the atomic bombings of Hiroshima and Nagasaki may be a biased population that does not represent the general population in several respects. Immunologically, human-leucocyte-antigen (HLA) allele frequency is a possible source of bias because during the acute phase of radiation sickness the immune function is usually depressed. Therefore, individuals bearing specific HLA alleles might have been selectively eliminated from the population due to these individuals' weak response to some types of pathogens. HLA class-II genes are involved in self- and non-self-recognition and are thus likely possible sources of selection among the survivors.

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Note: All authors are with the Department of Radiobiology, RERF.

In the past, HLA typing was done using a panel of antisera. Recent progress in molecular biology has shifted the approach toward direct DNA analysis.

Two methods, involving the polymerase chain reaction (PCR), that do not require subsequent direct sequencing are currently available for typing HLA class-II genes. One uses allele-specific-oligonucleotide (ASO) probes on PCR-amplified DNA (the PCR-ASO method)[1] (Amplitype, Cetus Corp, Emeryville, California), and the other involves the digestion of amplified DNA with restriction enzymes to separate DNA fragments by polyacrylamide gel electrophoresis [the PCR/restriction-fragment-length-polymorphism (RFLP) method].[2] However, the PCR-ASO method requires many different ASO probes corresponding to the polymorphic regions of each allele. The PCR-RFLP method, however, is associated with the complex electrophoretic band patterns found in heterozygous donors and requires several kinds of restriction enzymes for the identification of each allele.

Recently, single-strand-conformation-polymorphism (SSCP) analysis of DNA fragments amplified by the PCR method (PCR-SSCP analysis) was developed as a simple, sensitive method for the detection of DNA containing base-change mutations.[3] The rationale for this method is as follows: under nondenaturing conditions, single-strand DNA segments form sequence-specific conformations, and even a single-base substitution can alter the conformation, resulting in differences in the mobility of the DNA when it is subjected to polyacrylamide gel electrophoresis. Here we describe how an allele of the HLA-DQA1 locus could be completely typed using the combination of PCR-SSCP and single-restriction-enzyme cleavage.

Materials and Methods

Genomic DNA was extracted from peripheral blood or EBV-transformed B-lymphoblastoid cell lines and used as a template for PCR amplification. The HLA type of each sample was determined using PCR-ASO typing according to the method of Saiki et al[4] before use in our laboratory. Exon 2 of the HLA-DQA1 gene was amplified from 10 ng of genomic DNA in 10-mM Tris-HCl (pH 8.4), 1.5-mM $MgCl_2$, 50-mM KCl, 0.01% gelatin, 20-μM dNTP (each of the four types), 0.1-μM primer (both 5′-GTGCTGCAGGTGTAAACTTGTACCAG-3′ and 5′-CACGGATCCGGTAG-CAGCGGTAAGTTG-3′), and 1.0 μL of [α-^{32}P]dCTP (3000 Ci/mmol) in a 10-μL volume containing 0.2 units of Taq polymerase (Takara Co Ltd, Tokyo). Thirty-five PCR cycles were done with 95°C denaturation (30 s), 58°C annealing (1 min), and 72°C extension (1 min) in an automated thermal cycler. The PCR-amplified product was diluted 50-fold with 0.1% sodium dodecyl sulfate and 10-mM ethylenediaminetetraacetate (EDTA), followed by 1:1 dilution with 95% formamide, 20-mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. DNAs were heat denatured at 95°C for 10 min and then loaded onto a 20% nondenaturing acrylamide gel (30:1 ratio of acrylamide to methylene-bis-acrylamide, 46 × 16 × 0.1 cm, 0.5 cm per lane) containing 90-mM Tris-borate (pH 8.3) and 2-mM EDTA. Electrophoresis was carried out at 15 mA constant current for 16 h at 10°C (glass surface temperature), because resolution of the bands was greatly improved by cooling. After electrophoresis, the gel was transferred onto filter paper and dried. Overnight exposure of film at –80°C with an intensifying screen

was usually sufficient to reveal the signals. Furthermore, PCR-amplified DNAs cleaved by HphI were subjected to electrophoresis in 12% polyacrylamide gel. Restriction fragments were detected by staining with ethidium bromide.

Results

HLA-DQA1 has been classified into eight alleles: DQA1*0101, DQA1*0102, DQA1*0103, DQA1*0201, DQA1*0301, DQA1*0401, DQA1*0501, and DQA1*0601. Figure 1 shows the positions of the PCR primers used in the present study and the allelic variations in the 242- or 239-bp PCR-amplified regions of the HLA-DQA1 locus.[5,6]

The results of PCR-SSCP analysis of the homozygous DQA1 alleles extracted from each cell line showed eight pairs of unique bands (Figure 2) that were subsequently used as identification markers. Figure 3 shows the results of PCR-SSCP analysis of the blood from the 11 individual heterozygous donors. The HLA-DQA1 genotypes were identified using the reference bands of each homozygous DQA1 allele.

In the PCR-SSCP analysis of the eight homozygous alleles, two sets of pairs (DQA1*0103/DQA1*0301 and DQA1*0401/DQA1*0601) (lanes 3 and 5 and lanes 6 and 8, respectively, in Figure 2) showed similar mobilities in each respective set and therefore were difficult but not impossible to distinguish. As shown in Figure 2, lanes 3 and 5 or lanes 6 and 8 appear the same but are in fact not identical, as seen by the slight yet perceptible differences in the position of the lower bands. In some heterozygous cases, eg, lanes 3, 4, 6, 7, and 9 in Figure 3, such small differences may not always be distinguishable by PCR-SSCP alone. However, combination with an enzyme-cleavage assay eliminates any questions of allele identity. Figure 4 shows the patterns cleaved with a restriction enzyme, HphI, of the homozygous and heterozygous alleles. The DNAs of DQA1*0103, DQA1*0201, and DQA1*0601 were cleaved by HphI to produce fragments of 200, 197, and 197 bp, respectively. Use of the enzyme-cleavage assay resolved the problems mentioned above, namely, the bands representing DQA1*0103 and DQA1*0601, which were difficult to distinguish by PCR-SSCP analysis from DQA1*0301 and DQA1*0401, respectively, were cleaved and well distinguishable from each other (Figure 4a), as shown by the separation of the lower bands. In the case of heterozygous alleles (Figure 4b), the upper bands seen in several of the lanes are the result of heteroduplicity of the alleles, which varied in size. Consequently, the use of both PCR-SSCP and enzyme cleavage can segregate by either method alone difficult-to-separate samples.

We confirmed that the silver-staining method of detection was as effective as the radioisotope-labeling method to differentiate the PCR-SSCP gel bands. Moreover, under the assay conditions described, our results were reproducible.

Discussion

In this study, we developed a method for quick, simple genotyping of multiple samples of DQA1 alleles. In the PCR-SSCP analysis of HLA-DQA1 genotypes, electrophoresis with low concentrations of the DNA samples and at low gel surface temperatures improved the resolution. Moreover, because we were able to differentiate between DQA1*0101 and DQA1*0102, which differ by only one

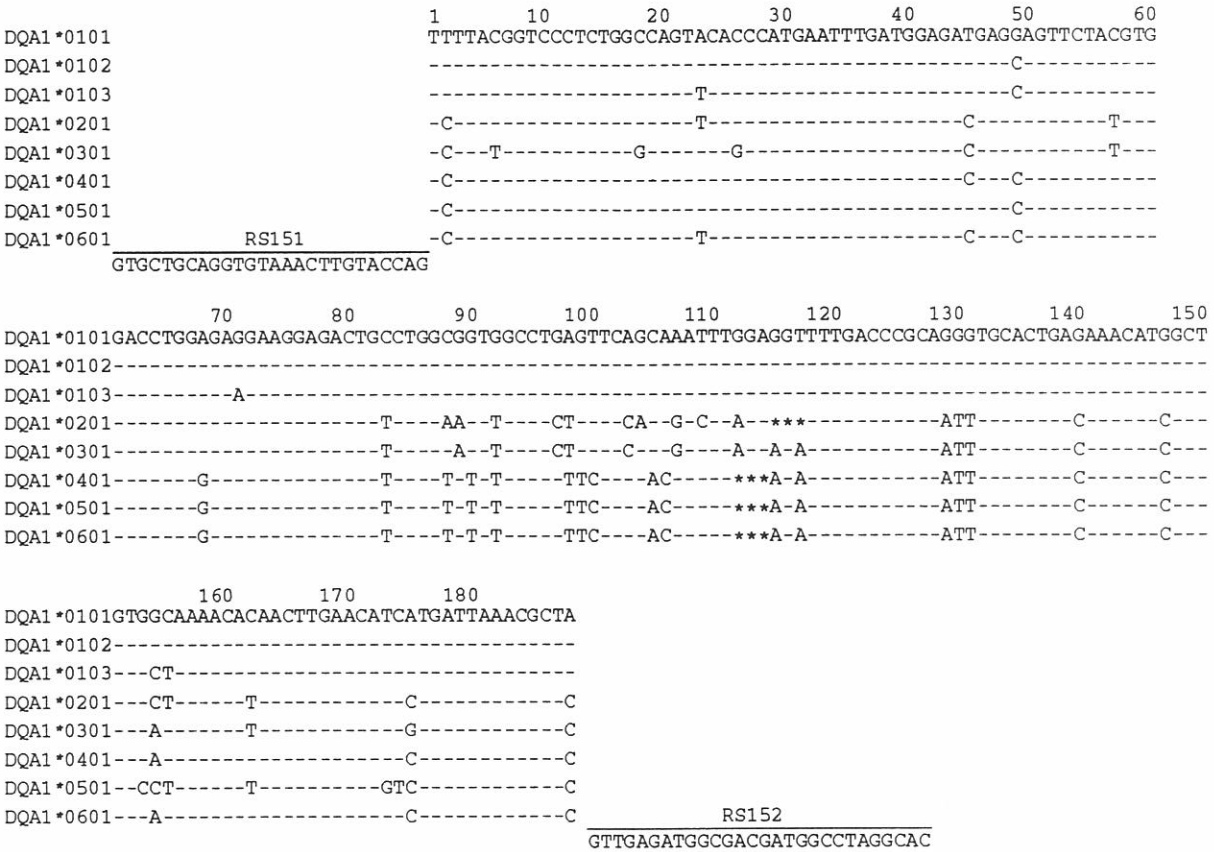

Figure 1. Alignment of the nucleotide sequences of the polymerase-chain-reaction-amplified exon-2 region in the DQA1 gene. The local DQA1 allele designations are made according to the official World Health Organization Nomenclature Committee. Nucleotide sequences of DQA1 alleles were taken from the following references: DQA1 *0201 and DQA1 *0301, Moriuchi et al,[5] and the others, Gyllensten and Erlich.[6] The star (*) indicates a 1-base deletion.

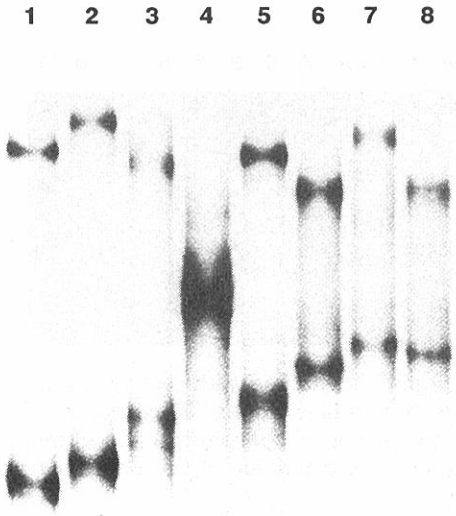

Figure 2. Single-strand-conformation-polymorphism analyses of polymerase-chain-reaction-amplified DNA of the homozygous HLA-DQA1 alleles extracted from each cell line (names are in parentheses). DNA samples were loaded onto 20% polyacrylamide gels for electrophoresis. Lane 1: DQA1*0101 (KAS116); lane 2: DQA1*0102 (SCHU); lane 3: DQA1*0103 (TOK); lane 4: DQA1*0201 (PLH); lane 5: DQA1*0301 (WT51); lane 6: DQA1*0401 (BTB); lane 7: DQA1*0501 (RML); lane 8: DQA1*0601 (LUY).

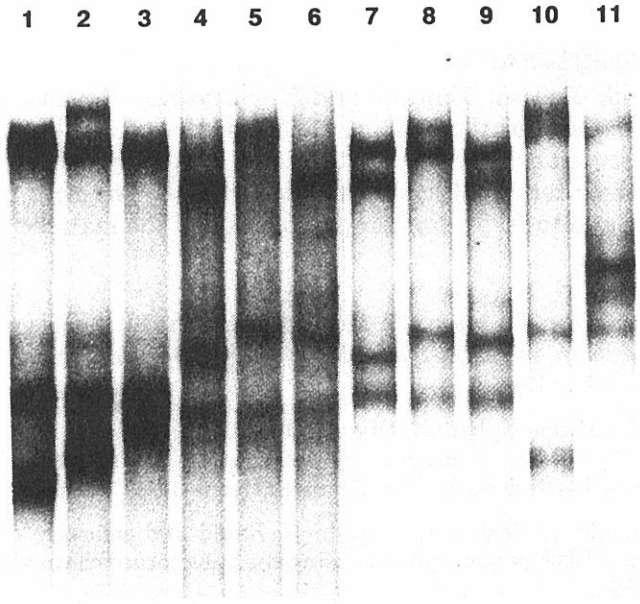

Figure 3. Single-strand-conformation-polymorphism analyses of polymerase-chain-reaction-amplified DNA of the HLA-DQA1 locus for 11 heterozygous individuals. DNA samples were loaded onto 20% polyacrylamide gels for electrophoresis. Lane 1: DQA1*0101/DQA1*0301; lane 2: DQA1*0102/DQA1*0301; lane 3: DQA1*0103/DQA1*0301; lane 4: DQA1*0401/DQA1*0103; lane 5: DQA1*0501/DQA1*0103; lane 6: DQA1*0601/DQA1*0103; lane 7: DQA1*0301/DQA1*0401; lane 8: DQA1*0301/DQA1*0501; lane 9: DQA1*0301/DQA1*0601; lane 10: DQA1*0102/DQA1*0501; lane 11: DQA1*0201/DQA1*0501.

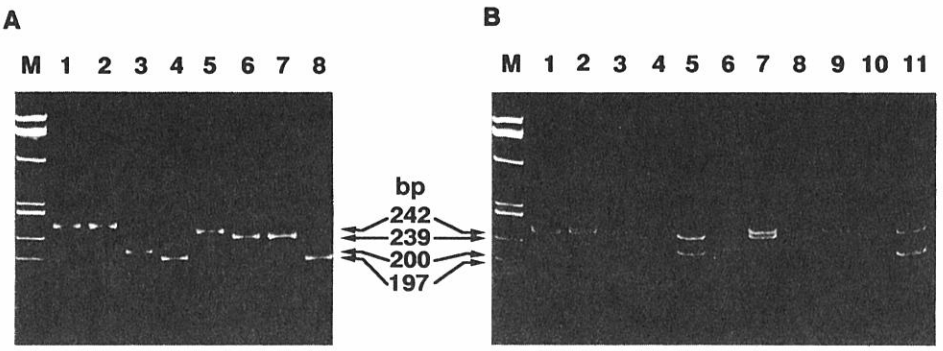

Figure 4. Patterns of polymorphic restriction fragment obtained with HphI-restriction-enzyme digestion of HLA-DQA1 homozygous (A) and heterozygous (B) samples. DNA samples were loaded onto 12% polyacrylamide gels for electrophoresis. Lane numbers of A and B correspond to those in Figures 2 and 3, respectively. Size markers from HaeIII-digested ϕX-174 DNA are found in lane M.

nucleotide (G-C at position 49 in Figure 1), this method should also be useful for detecting new DQA1 genotypes as well as point mutations. Currently, attempts are underway to develop this new method further to allow typing of other HLA genes (HLA-B, -DQB1, -DPB1, and -DRB1).

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