Simultaneous Analysis of Two-Site Gene Polymorphisms Using Time-Resolved Fluorescence Immunoassay

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We have developed a method for the simultaneous analysis of two-site gene polymorphisms. The principle developed by us is that two amplicons are produced by duplex PCR using two different primer sets (digoxigenin labeled primer A and biotinylated primer B for BsmI recognition site, biotinylated primer C and FITC labeled primer D for TaqI recognition site) and digested with two restriction enzymes, and the reaction products are analyzed by time-resolved fluorescence immunoassay (TR-FIA) using two different lanthanide ions (Eu and Sm) as labels. In this study, BsmI and TaqI recognition site polymorphisms in intron 8 and exon 9 of the vitamin D receptor (VDR) gene were selected as a target gene. VDR gene polymorphisms of 27 subjects were determined by the proposed method. The results agreed with that obtained by a restriction fragment-length polymorphism (RFLP) analysis using gel electrophoresis. The proposed method is effective, has excellent precision and is well suited to gene analysis for a large number samples.

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Gene diagnosis is carried out by the analysis of an amplified specific gene product using the polymerase chain reaction (PCR).¹ PCR techniques have been used for the direct detection of specific genes in clinical specimens, such as blood, urine and tissues. Also, multiplex PCR that amplify plural gene simultaneously has been used in microbiology.^{2,3}

On the other hand, the analysis of a variant gene and gene polymorphism can be carried out by PCR-restriction fragment length polymorphism (RFLP) or PCR-single strand conformation polymorphism (SSCP). Although, in many case, PCR or following steps, such as the digestion of RFLP or the denaturation of SSCP, is usually completed within several hours, the analysis of these end products using an agarose gel electrophoresis step requires much time. This process limits the number of assays that can be completed within the working times, and complicates the introduction of PCR or PCR-RFLP, SSCP into clinical routine diagnosis.

In recent years, easy and rapid detection methods of PCR products using hybridization^{4,5} and immunochromatography (IC)^{6,7} have been developed, though there are several problems: for instance, the hybridization method requires detection step by a probe and a denature step; also IC is not sensitive, dose not have a high throughput, and is not suitable for the analysis of gene polymorphism.

We previously developed a bioluminescent enzyme-linked immunosorbent assay combined with RFLP.⁸ The method enabled a fast detection and sensitive assay of PCR products. However, a more useful method, such as the simultaneous analysis of two-site gene polymorphism is also required in the clinical field. Therefore, we designed a method that uses a 96-well microtiter plate as a test module and a time-resolved fluorescence detection system. We selected lanthanide ion chelates as labeling materials, because of their characteristics, such as long-time luminescence that causes a decrease in the background fluorescence, having a large Stokes' shift and sharp the emission wavelength depend on lanthanides. These

characteristics lead the development of the assay having highly sensitive and simultaneous detection assays. Particularly, a simultaneous assay has many advantages over the sequential methods in terms of the diagnosis efficiency.

In 1994, the relationship between bone turnover and the vitamin D receptor (VDR) gene polymorphism in Caucasians was reported.⁹ In the report, allelic variations of BsmI RFLP in intron 8 correlate with the serum osteocalcin concentration and bone mineral density (BMD). Moreover, Tokita *et al.* examined the allelic influences on bone turnover using combined BsmI RFLP with Apal and TaqI RFLPs in intron 8 and exon 9.¹⁰ As a result, the effect of VDR alleles on BMD was found to be similar to that obtained in Caucasians, and can be more discriminated to a subgroup using multiple RFLPs.

Measurements of various components in plasma have been performed¹¹⁻¹⁴ using a rare earth as a reporter molecule. However, there are a few reports related to gene analysis.^{5,15} In this study, we describe the development of a simultaneous detection method for the measuring the duplex PCR-RFLP products. Thus, the BsmI recognition site and the TaqI recognition site on the VDR gene were analyzed by time-resolved fluorescence immunoassay (TR-FIA) with two different lanthanide ion-chelate labels.

Experimental

Instruments

Polymerase chain-reaction experiments and endonuclease digestion were carried out in a GeneampTM PCR system 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The microtiter plate washer Model AMW-8 was from BioTec Co. (Tokyo, Japan). Immunoassays were performed using an Amerlite shaker/incubator (Amersham, Oakville, ON, Canada). Time-resolved fluorescence detection was carried out using a DELFIATM 1234 fluorometer (Wallac Oy, Turku, Finland).

Table 1 Primer sequence used for duplex PCR

Primer name	Label (5' end)	Sequence	Target	Product size (bp)
A (Forward)	Digoxigenin	5'-CCCTTAGCTCTGCCTTGCA	BsmI region	273
B (Reverse)	Biotin	5'-AACCAGCGGAAGAGTCAAGGG	BsmI region	
C (Forward)	Biotin	5'-CAGAGCATGGACAGGGAGCAAGGCCA	TaqI region	321
D (Reverse)	FITC	5'-TGTGTTGGACAGGCGGTCT	TaqI region	

Reagents

The 5' end labeled origonucleotide primers used in PCR were synthesized by Takara Shuzo Co. (Kyoto, Japan). A set of primers to generate a 273bp product for the BsmI recognition site were labeled with biotin and digoxigenin, respectively. Similarly, primers to generate a 321bp product for the TaqI recognition site were also labeled with biotin and FITC, respectively (Table 1). Restriction endonuclease BsmI and TaqI were purchased from New England Biolabs Inc. (Beverly, MA, USA) and Nippon Gene Co. (Toyama, Japan), respectively. A Maxisorp microtitration plate made of polystyrene was purchased from Nunc (Roskilde, Denmark). Rabbit monoclonal anti-FITC IgG and sheep monoclonal anti-digoxigenin IgG were supplied by Roche diagnostics Inc. (Basel, Switzerland) and DAKO (Glostrup, Denmark), respectively. A DELFIATM assay buffer and an Enhancement solution, europium(III) chelated with *N*¹-(*p*-isothiocyanatebenzyl)-diethylene-triamine-*N*¹,*N*²,*N*³,*N*³-tetraacetic acid and samarium(III) chelated with *N*¹-(*p*-isothiocyanatebenzyl)-diethylene-triamine-*N*¹,*N*²,*N*³,*N*³-tetraacetic acid, were from Wallac Oy (Turku, Finland). Avidin and diethylenetriamine-*N*,*N*,*N*¹,*N*²,*N*²-pentaacetic acid (DTPA) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Blockace was from Dainippon Pharmacy Co. (Osaka, Japan). A post-coating solution (0.1 M PBS (pH 7.0) containing 0.1% sodium azide, 5 mM DTPA and 50% Blockace) and washing solution (50 mM Tris-HCl (pH 7.0) containing 0.05% Tween20 and 0.9% NaCl) were prepared in house. All other chemicals used were of analytical grade.

Specimen preparation

DNA from peripheral blood mononuclear cells of 27 subjects were obtained by a standard method using digestion with proteinase K and extraction with phenol-chloroform, followed by ethanol precipitation. The obtained genomic DNA was dissolved into TE buffer and stored at -40°C until use. These genomic DNA samples with informed consent were supplied from Dr. A. Tokita of Juntendo University.

PCR experiments

A 273bp product with a BsmI recognition site and a 321bp product with a TaqI recognition site, in intron 8 and exon 9 of a gene-encoding human vitamin D receptor, respectively, were simultaneously amplified by duplex PCR using each primer set. Amplification was carried out in a total volume of 25 µL solution containing (final concentration) 100 ng human genomic DNA or distilled water for negative control, 0.2 mM of each four deoxynucleotide triphosphates (dATP, dGTP, dCTP and dTTP), 0.2 µM of four labeled primers and 0.75 U of Taq polymerase (Roche diagnostics Inc.) in a reaction buffer (10 mM Tris-HCl, pH 8.3, containing 1.5 mM of MgCl₂, 50 mM of KCl and 5% of DMSO). Amplification reactions were performed at the following temperatures and times: first, denaturation at 94°C for 1.5 min, followed by denaturation at 94°C for 0.5 min, annealing and extension at 64°C for 1.5 min. After 33 times of thermal cycling, in order to complete the extension, the final

extension step was performed at 72°C for 7 min and then cooled to 4°C. PCR products were stored at -40°C until use.

RFLP experiments

An RFLP analysis was carried out using restriction enzymes, BsmI and TaqI, to screen for any mutations in intron 8 and exon 9 on the VDR gene, respectively. Five microliters of PCR products were added to 5 µL of a restriction buffer (final concentration: 0.6 mM Tris-HCl, pH 7.9, 15 mM NaCl, 0.6 mM MgCl₂ and 0.1 mM dithiothreitol) containing 3 U of BsmI, 4 U of TaqI. Digestion was carried out for 2 h at 65°C in a thermal cycler. After that, those samples with or without digestion were analyzed on agarose gel electrophoresis, followed by ethidium bromide staining and proposed time-resolved fluorescence immunoassay. In electrophoresis, BsmI digestion in high BMD subjects resulted in two restriction fragments of 177bp and 96bp. If BMD decrease, three fragments (273bp, 177bp and 96bp) were observed in heterozygous, whereas no digestion occurred in homozygous of low-BMD subjects, showing only 273bp fragment. The corresponding pattern after TaqI digestion in high-BMD subjects was only one band of 321bp (no digestion). Three fragments (321bp, 291bp and 30bp) were shown in heterozygous subjects. Also, the homozygous of low-BMD subjects showed 291 and 30bp fragments.

Time-resolved fluorescence immunoassay experiments

(a) Coating microtiter plate with avidin

A microtiter plate was coated with 200 µL of a 20 mg/L solution of avidin in a 50 mM sodium bicarbonate buffer (pH 9.5) overnight at room temperature. After coating, the avidin solution was removed. A 200-µL volume of a post-coating solution was added to each well and then stored at 4°C until use.

(b) Preparation of immunoglobulin labeled with europium (Eu) and samarium (Sm)

The labeling of monoclonal IgG was performed as previously described.¹¹ Anti-FITC IgG was labeled with the Eu chelate. The other monoclonal anti-digoxigenin IgG was conjugated with the Sm chelate. In brief, 1 mg of IgG was labeled with a 45-fold (Eu) or 30-fold (Sm) molar excess of lanthanide ion chelates in a 50 mM sodium bicarbonate buffer, pH 9.5, and subsequently incubated overnight at room temperature. The labeled IgG was purified by gel filtration on a 1.5×45 cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 50 mM Tris-HCl (pH 7.0) containing 0.05% NaN₃ and 0.9% NaCl. We collected 1.0 mL of fractions and measured their absorbance at 280 nm for determining the IgG mass. The labeling degrees of each fraction containing IgG were determined using standard solutions of Eu and Sm by time-resolved fluorometry. The elution profile of each lanthanide labeled IgG are shown in Fig. 1. The labeled anti-FITC IgG show 15 Eu molecules per IgG and the labeled anti-digoxigenin IgG also was 17 Sm molecules per IgG, respectively. We used a fraction with a high IgG concentration and high fluorescence intensity as a conjugate.

(c) Immunoassay procedure

Fig. 1 Sephadex G-25 column elution profiles of lanthanide ion chelate-labeled IgG. Graph A is the elution profile of Eu-labeled anti-FITC IgG and graph B of Sm labeled anti-digoxigenin IgG, respectively. The open circles (○) indicate the absorbance at 280 nm of each IgG and closed circles (●) indicate the fluorescence intensity of each lanthanide chelate, respectively.

A simultaneous analysis using TR-FIA was carried out in an avidin-coated microtiter plate. The prepared avidin-coated plate was washed three times with a washing buffer before the assay. PCR products without digestion and PCR-RFLP products with digestion were diluted 50 times or 25 times with the assay buffer, respectively. A 100-μL volume of each diluted sample solution was dispensed into separate microwells, followed by 100 μL of a conjugate solution (×500) containing both Eu-labeled anti-FITC IgG and Sm-labeled anti-digoxigenin IgG being added. Incubation was carried out for 2 h at 37°C with continuous shaking. After 6-times washing, 200 μL of an enhancement solution was added into each well. The plate was then shaken for 5 min at room temperature and the fluorescence intensities from the Eu and Sm chelate were measured by dual detection program on a 1234 DELFIA™ fluorometer. The total-assay principle is schemed in Fig. 2.

(d) Determination of gene polymorphism

Gene polymorphism was determined based on the non-restriction percent, which was calculated from the ratio of the fluorescence intensity (FI) from samples before and after digestion. The formula used was

$$\text{Non-restriction percent (\%)} = \frac{\text{FI with digestion}}{\text{FI without digestion}} \times 100$$

Results and Discussion

Because PCR is good tool for the selective and exponential amplification of specific gene sequences, it has been used in DNA diagnosis. Generally, the detection of one-point mutation on a specific gene is carried out by RFLP or SSCP analysis

Fig. 2 Principle of PCR-RFLP-TRFIA. After duplex PCR with the four primer (A, B, C and D), the products were treated with two restriction enzymes (BsmI and TaqI), simultaneously. According to the genotype, the DNA duplexes were digested. After digestion, the obtained DNA fragments were immobilized by their biotin end onto an avidin-coated microtiter plate. Detection was carried out by the addition of Eu-labeled anti-FITC IgG and Sm-labeled anti-digoxigenin IgG. The labeled IgG were allowed to bind simultaneously to their corresponding labeled haptens, FITC and digoxigenin. The labeled lanthanide ions were released and re-chelated by the addition of an enhancement solution. Then, the Eu and Sm fluorescence intensity were measured.

combined with PCR. However, the analysis is complicate and not suitable for an automatic method. The proposed method using time-resolved fluorescence detection with two lanthanide ion chelates, such as Eu and Sm, allows the simultaneous determination of PCR-RFLP products in two PCR products. In this study, the VDR gene related with bone turnover was selected as a target gene.

Optimization of simultaneous PCR-RFLP analysis using duplex PCR

Two pairs of primers were designed from the sequenced DNA in intron 8 and exon 9 of human VDR locus. The four primers are listed in Table 1. The PCR-RFLP analysis was optimized by using 3 genotypes of BBtt, BbTt, and bbTT, respectively. The following parameters were examined to optimize the PCR-RFLP analysis: annealing and extension temperature, thermal cycling number, restriction enzyme concentration and incubation time.

The annealing and extension temperatures were varied from 62 to 68°C in 35 cycles. When the temperature was higher than 65°C or below 63°C, there is no suitable BsmI region PCR products for the proposed assay. However, the amounts of TaqI region PCR product was not changed under these various temperature conditions. Therefore, we determined that optimum annealing and extension temperatures for the duplex PCR are 64°C.

The thermal cycling numbers used for the assay was varied from 30 to 35 cycles at 64°C. When the thermal cycling number was less than 32 cycles, the amount of BsmI region PCR product decreased. When increasing the thermal cycling numbers, the Eu background increased, whereas the PCR product contents did not increase. Therefore, we selected 33

cycles in a following experiment.

The effects of the restriction enzyme concentration and incubation time on the RFLP analysis were examined using a 5 μ L PCR product solution in a volume of 10 μ L. The enzyme concentration was varied from 1 to 7 U for BsmI, and from 2 to 15 U for TaqI, respectively. When the concentration of the restriction-enzyme was greater than 3 U for BsmI and 4 U for TaqI, the digestion efficiency remained constant. Next, the incubation time was varied from 1 to 5 h using 3 U BsmI and 4 U TaqI. When the incubation time was longer than 2 h, no

increase of digestion efficiency was observed.

The optimum condition for the duplex PCR-simultaneous RFLP procedure with the VDR locus was therefore determined as follows: The thermal cycling profile was 94°C for 0.5 min, and 64°C for 1.5 min. Thirty three cycles were found to be optimal. RFLP mixture was contained with 3 U of BsmI, 4 U of TaqI, 0.6 mM Tris-HCl (pH 7.9), 15 mM NaCl, 0.6 mM MgCl₂ and 0.1 mM DTT (each final concentrations), and 5 μ L of duplex PCR solution in a final volume of 10 μ L. Digestion was carried out for 2 h at 65°C.

Optimization of time-resolved fluorescence immunoassay

The effects of various factors, such as the avidin concentration coated on a microtiter plate well, the Eu- and Sm-labeled antibody concentrations, and the incubation time, were examined to optimize TR-FIA. First, the avidin concentration was varied from 5 to 40 mg/L. Increasing the avidin concentration also resulted in an increase of the fluorescence intensity. More avidin than 20 mg/L gave a high fluorescence intensity. Subsequently, the dilution ratio of the labeled antibody, the PCR product and the PCR-RFLP product were varied from 100 fold to 4000 fold as a final concentration, respectively. In the result, high fluorescence intensity and a high signal-to-noise ratio were obtained with the condition of 1000-fold diluted labeled antibody and of 100-fold diluted PCR or PCR-RFLP product. The results are indicated in Fig. 3. The incubation time was varied from 0.5 h to 6 h. From this result,

Fig. 3 (A) Effect of the PCR product dilution ratio on the fluorescence intensity. (B) Search for a suitable dilution ratio of lanthanide-labeled IgG.

Table 2 Assay precision of simultaneous TR-FIA

Sample		BsmI recognition site		TaqI recognition site	
		Sm Mean ^a $\pm$ SD	CV, %	Eu Mean ^a $\pm$ SD	CV, %
Within-run (n=8)	A	2882 $\pm$ 64	2.22	124639 $\pm$ 1430	1.15
	B	4300 $\pm$ 173	4.04	168539 $\pm$ 2502	1.49
	C	5451 $\pm$ 191	3.51	184722 $\pm$ 5211	2.82
Between-run (n=7)	A	2937 $\pm$ 97	3.32	104919 $\pm$ 1757	1.68
	B	4471 $\pm$ 153	3.43	144028 $\pm$ 2158	1.50
	C	5936 $\pm$ 214	3.60	164296 $\pm$ 2741	1.67

a. The data were indicated by fluorescence intensity.

Table 3 Determination of VDR gene polymorphism on TaqI and BsmI region by the proposed method

Sample number	TaqI non-restriction, %	BsmI non-restriction, %	Sample number	TaqI non-restriction, %	BsmI non-restriction, %
1	98.6	18.6	17	40.1	50.4
2	98.5	13.7	18	50.0	83.2
3	99.7	17.8	19	41.6	49.1
4	94.6	23.6	20	44.6	48.7
5	102.1	17.1	21	47.5	79.0
6	98.4	19.5	22	42.7	45.6
7	98.7	14.8	23	46.4	72.4
8	98.1	7.7	24	48.2	75.0
9	97.3	17.0	25	43.1	50.6
10	97.7	20.1	Mean	44.9	61.6
11	97.2	0.1			
12	96.3	0.8			
13	92.3	6.1			
14	87.5	19.6			
15	93.0	2.1	26	10.8	103.1
16	91.2	2.6	27	0.7	101.1
Mean	96.3	12.6	Mean	5.7	102.1

No.1 - No.16 were TTbb genotype. These genotypes were determined by RFLP analysis using agarose gel electrophoresis. No.17 - No.25 were TtBb genotype. No.26 and 27 were ttBB genotype.

Fig. 4 (A) Agarose gel electrophoresis of PCR-RFLP products amplified from 3 typical genotypes (T1 (TTbb), T2 (TtBb) and T3 (ttBB), respectively). Lanes: M, ϕ X174/Hae III digest; N, negative control; T1, TTbb genotype; T2, TtBb hetero genotype; T3, ttBB genotype, respectively, amplified with primers A, B, C and D. (B) Zymogram of four discordant samples were shown. Density of 273bp product indicated by an arrow was stronger than that of typical hetero genotype (lane T2 in A).

we determined that a optimal incubation time is 2 h which was used in the following experiments.

Precision study

To evaluate the within- and between-run precision of the simultaneous TR-FIA, three samples containing both BsmI region amplicon and TaqI region amplicon were measured. The results are summarized in Table 2. The CV% values were 1.15 to 4.04% in within-run precision, and 1.50 to 3.60% in between-run precision, respectively. These results mean that the proposed method is an excellent dual assay in terms of precision.

Application to clinical samples

Genotype classifications of 27 samples were determined by the proposed method. The results are summarized in Table 3. The results determined by gel electrophoresis are also presented in Table 3. As a result, in the TaqI region the non-restriction percents were 87.5 – 102.1 for TT genotype, 40.1 – 50.0 for Tt genotype, 0.7 – 10.8 for tt genotype, respectively. On the other hand, in the BsmI region they were 101.1 – 103.1 for BB genotype, 49.1 – 83.2 for Bb genotype, 0.1 – 23.6 for bb genotype, respectively. The non-restriction percent of homozygous showed values of 100 or 0 neighborhood, and almost heterozygous showed about 50%. However, in the case of four samples (18, 21, 23 and 24) which were a heterozygous sample, the non-restriction percents of the BsmI region polymorphism were 83, 79, 72 and 75, respectively. It is thought that these results were caused by DNA conformation produced by amplification and haptens used as a label. Therefore, the results of the proposed method were confirmed by gel electrophoresis to determine whether the BsmI enzyme works well under the proposed condition. The fluorescence intensity of each DNA fragment on agarose gel was measured by an image scanner, and the results of the non-restriction percent from gel were 74, 71, 85 and 68, respectively (Fig. 4). The results show that the result of TR-FIA is well related with that of gel electrophoresis. Thus, the proposed method well reflects RFLP analysis by gel electrophoresis.

Conclusion

In this paper, we have discussed a simultaneous analysis method of two-site polymorphism of the VDR gene which are recognized by two restriction enzymes. As a detection system, a time-resolved fluorescence detection method using a rare earth and immunoreaction was used. The simultaneous two-site polymorphism detection of the VDR gene which was developed by us was shown to be efficient and rapid compared with gel electrophoresis. Also the specificity corresponded to that of RFLP analysis using gel electrophoresis. Furthermore, the proposed method is useful for the analysis of a large number samples. It will greatly contribute to the labor-efficiency of a future gene diagnosis and facilitate its introduction to a clinical laboratory test.

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