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SHORT COMMUNICATION

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SNP genotyping in the β_2-adrenergic receptor by electronic microchip assay, DHPLC, and direct sequencing

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Abstract The β_2-adrenergic receptor (β2AR) is the key target for the β_2-agonist drugs used for bronchodilation in asthma and chronic obstructive pulmonary disease. To detect four SNPs with amino acid variations at positions −47T/C (CysBUP19Arg), 46A/G (Gly16Arg), 79C/G (Gln27Glu), and 491C/T (Thr164Ile) in the β*2AR* gene, we used the electronic microchip assay, denaturing high-performance liquid chromatography (DHPLC), and direct sequencing. Genomic DNA samples were obtained from the blood of 84 Japanese healthy volunteers. The agreement rates of the first data set with the final data (allele calls) were 99.7% (332/333), 99.2% (246/248), and 96.7% (329/340). The percentages of no allele designation (ND) were 2.06% (7/340), 2.75% (7/255), and 0.00% (0/340) for the electronic microchip assay, DHPLC, and direct sequencing, respectively. Furthermore, we found three samples that had a novel haplotype.

Key words Single-nucleotide polymorphisms (SNPs) · β_2-Adrenergic receptor (β2AR) · Electronic microchip · DHPLC · Direct sequencing

Introduction

The β_2-adrenergic receptor (β2AR) is a G-protein-coupled cell surface receptor that is the key target for the β_2-agonist drugs used as bronchodilators and β-blockers **(**Liggett 2000**)**. The relation between polymorphism of the β2AR receptor and therapeutic response has been reported (Erickson and Gravis 2001; Ligget 1997; Makimoto et al. 2001). In the human population, nonsynonymous single-nucleotide polymorphisms (SNPs) at positions −47T/C (CysBUP19Arg), 46A/G (Gly16Arg), 79C/G (Gln27Glu), and 491C/T (Thr164Ile) in the β*2AR* gene and three haplotypes on the prescribed SNPs have been identified (Drysdale et al. 2000). In this study, we conducted genotyping of these four SNPs using the electronic microchip assay, DHPLC, and direct sequencing to evaluate which method is applicable in the clinical setting.

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Subjects and methods

Genomic DNA samples were obtained from the blood of 84 Japanese healthy volunteers and prepared from a Blood and Cell Culture Kit (Qiagen, Hilden, Germany). Ten nanograms of genomic DNA was amplified by PCR with 0.5 μM of primer in 25 μl of standard reaction buffer for 35 cycles of 30 s each at 94°C and 65°C, with a 30-s extension at 72°C. Primer sequences were as follows: F1,5′-CTG AGT GTG CAG GAC GAG TC; R1,5′-AAG TAG TTG GTG ACC GTC TGC AG; F2,5′-TTC CAG GCG TCC GCT CG; R2(′),5′-(biotin)-ATG GCC AGG ACG ATG AGA G; F3,5′-CTG CAG ACG GTC ACC AAC TAC T; F4,5′-AAG A AT AAG GCC CGG GTA AT; R3, 5′-GAA GAG GCA ATG GCA TAG GCT (Fig. 1). The instrument for the electronic microchip assay was the NanoChip Molecular Biology Workstation (Nanogen, San Diego, CA, USA) (Gilles et al. 1999; Sosnowski et al. 1997). The electronic microchip (NanoChip cartridge) contains 100 test sites. The Cy3- and Cy5-labeled reporter oligonucleotides (R), the biotin-labeled capture oligonucleotide (C), and the stabilizer oligonucleotide (S) were designed as follows: (−47S),5′-GCT GAG GCG CCC CCA GCC AGT GCG CT; (−47R), 5′-Cy3- GGG TCC GCC T/5′-Cy5-GGG TCC GCC C; (46S), 5′-GAA GCC ATG CGC CGG ACC ACG ACG TCA C; (46R), 5′- Cy3-GGC ACC CAA TA/5′-Cy5-GCA CCC AAT A; (79S), 5′-AAA GGG ACG AGG TGT GGG TGG TGG GCA; (79R), 5′-Cy3-TCA CGC AGC/5′-Cy5-TCA CGC AGG; (491C), 5′-CTC CTT CTT GCC CAT TCA GAT GCA CTG GTA C; and (491R),

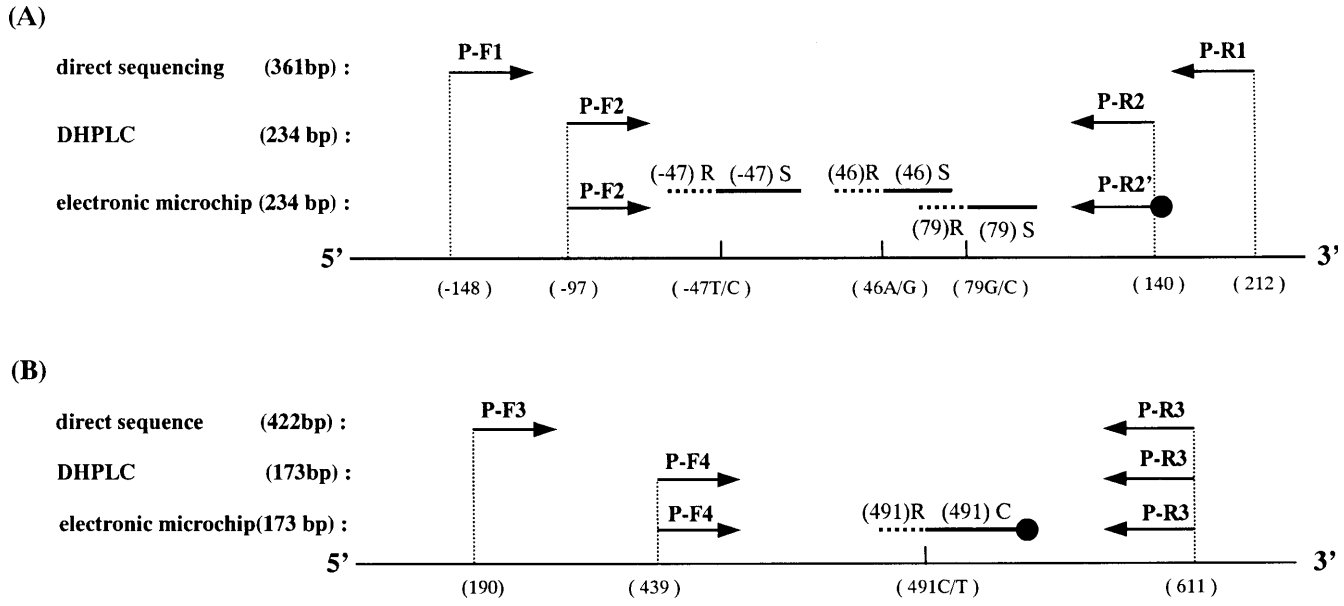

Fig. 1A,B. Oligo design for the electronic microchip assay, denaturing high-performance liquid chromatography (DHPLC), and direct sequencing. **A** The design for −47T/C, 46A/G, and 79C/G. **B** The design for 491C/T. The *closed circles* indicate biotin. The *arrows* indicate polymerase chain reaction primer (P-F1–4: forward primer, P-R1–3: reverse primer). The *dotted lines* (R-1–4) the *thick lines* (S-1–3), and adding biotin (C-1) indicate reporter, stabilizer, and capture oligos for the electronic microchip assay. The nucleotide numbers of the β2*AR* gene are according to GenBank (M15169). The four single-nucleotide polymorphism sites of this figure refer to the first nucleotide of the open reading frame as nucleotide (1) (corresponding to nucleotide 1588 of M15169), with the 5′-untranslated region beginning at −1 and proceeding in the negative direction. The sequences of oligos are mentioned in Table 1

5′-Cy3-GTC AGG CCT TAC/5′-Cy5-TCA GGC CTT AT (Fig. 1). Each PCR product was desalted using a multiscreen plate (Millipore, Bedford, MA, USA). The protocols were then run at predetermined temperatures on a NanoChip system according to the Nanogen application guide. Samples were scored automatically according to the following threshold: heterozygote < 1:2 < no designation < 1:5 < Homozygote. The DHPLC instrument was the WAVE system (Transgenomic, Omaha, NE, USA). Unpurified PCR amplicons were heated at 95°C for 5 min and cooled slowly over 45 min to 25°C in a thermocycler. The reactions were then run at predetermined temperatures on the WAVE system, as described (Shinka et al. 2001). The resultant chromatograms were compared for variation in shape and/or retention time. For direct sequencing, PCR products were purified using the QIA Quick PCR Purification Kit (Qiagen, Valenica, CA, USA) and were sequenced with an automated sequencer (377XL, Applied Biosystems, Foster City, CA, USA) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, as described (Kikuchi et al. 2000). Data were analyzed with Sequencher software (Hitachi Software, Tokyo, Japan). For positions −47T/C, 46A/G, and 79C/G, we conducted the electronic microchip assay, DHPLC, and direct sequencing methods until identical data were obtained for all three methods. The 491C/T genotyping was conducted by electronic microchip assay and direct sequencing. The agreement data between the systems were defined as the final data (allele call). The agreement rate was calculated as the correspondence rates of the first data set with the final data (allele call).

Results and discussion

Figure 2 shows the 46A/G results by electronic microchip assay (A), DHPLC (B), and direct sequencing (C). The upper part of Fig. 2A shows a portion of the real-time analytic screen. If we selected #18 as the hetero control, the scoring was conducted automatically according to the previous threshold. Figure 2B and Fig. 2C show typical patterns obtained with DHPLC and direct sequencing. The agreement rates of the first data set with the final agreement data (allele calls) were 99.7% (332/333), 99.2% (246/248), and 96.7% (329/340). The percentages of no allele designation (ND) were 2.06% (7/340), 2.75% (7/255), and 0.00% (0/340) for the electronic microchip assay, DHPLC, and direct sequencing, respectively (Table 1). The DHPLC and direct sequence disagreement data were caused by human error in manual analysis. The total agreement ratio was the highest among the three tested methods. The 491C/T results by the electronic microchip assay agreed with those from direct sequencing, even in the presence of nonspecific PCR products. Therefore, the electronic microchip system would be a powerful tool for clinical use.

In the present study, we found three samples that had novel haplotypes, which was supported by the DHPLC result. The chromatography patterns of the samples by DHPLC disagreed with that of previously reported haplotypes (Drysdale et al. 2000). The putative haplotypes did not show linkage disequilibrium between −47T/C and 79C/G. The −47T/C SNP site is located at the β2AR regulation region and has been reported to show linkage disequilibrium with 79C/G. Parola and Kobilka (1994)

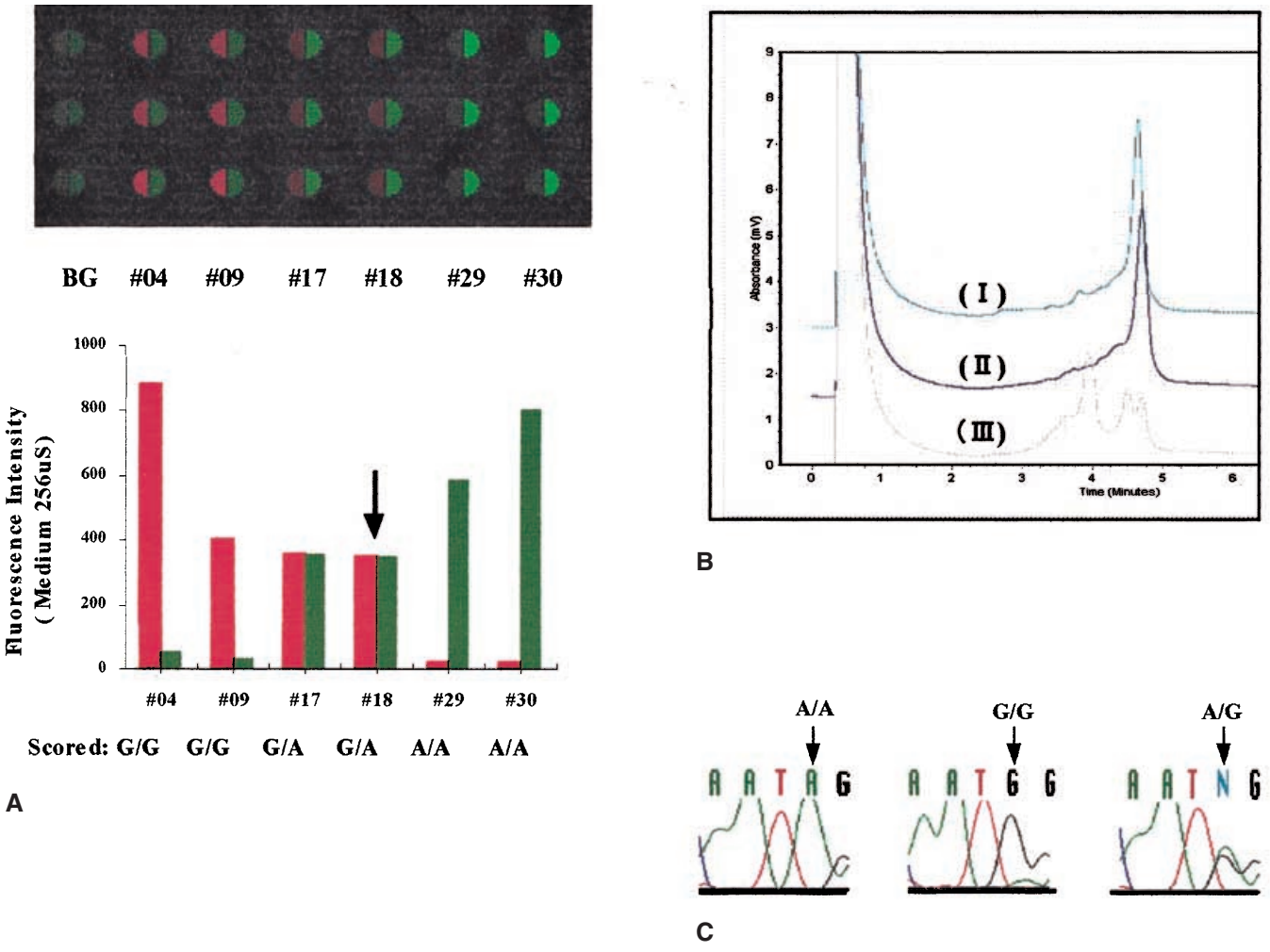

Fig. 2A–C. The results of 46A/G genotyping. **A** Electronic microchip assay results. The upper picture indicates a Cy3/Cy5 image of a representative chip. The lower figure indicates the result of quantification and scoring of random samples. *Red and green columns* show the Cy5 (G allele) and Cy3 (A allele) signal. The *arrow* was designated as the heterozygote control. Thermal stringency was conducted at 38°C. **B** DHPLC chromatogram patterns. The patterns of I, II, and III showed A/A (#02), G/G (#23), and G/A (#06), respectively. The column temperature was 65.5°C and the time shift was 1.5. **C** The chromatography patterns of direct sequencing. The *arrows* point at the 46A/G position. The *left*, *middle*, and *right figures* show A/A (#29), G/G (#04), and A/G (#17), respectively

Table 1. Total agreement and no designation (ND) ratio of first data on genotyping by electronic microchip assay, DHPLC, and direct sequence; frequency of each SNP; reported haplotype; and samples having novel haplotypes

	SNP agreement ratio of the first-run data				Total ratio (%)	
Method	−47T/C	46A/G	79C/G	491C/T	Agreement ratio	ND ratio
Electronic microchip	77/78	83/83	84/84	84/84	**99.7** (328/329)	**2.08** (7/336)
DHPLC	82/82	80/81	81/82	—	**99.2** (243/245)	**2.78** (7/252)
Direct sequence	79/84	81/84	81/84	84/84	**96.7** (325/336)	**0.00** (0/336)
Frequency of Japanese sample (%)	T: 97.1	G: 54.1	C: 93.5	C: 100.0		
Reported haplotype	T	A	C	C	**32.5**	
(Drysdale et al. 2000)	T	G	C	C	**57.5** } allel frequency (%)	
	C	G	G	C	**10.0**	
#11, 32, 76	T/C	A/G	C/C	C/C		

DHPLC, Denaturing high-performance liquid chromatography; SNP, single-nucleotide polymorphism

reported that the peptide product of a 5′ leader cistron in the β2AR mRNA inhibited receptor synthesis. McGraw et al. (1998) reported that the −47T/C variation may represent the genetic basis of variable physiologic sympathetic responses, variation in disease phenotypes, or differences in therapeutic efficacy of β-agonists or antagonists. Further studies are needed to prove that these three samples contain novel haplotypes. The relationship between the haplotype of β2AR and therapeutic response and disease should also be investigated.

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