A microarray minisequencing system for pharmacogenetic profiling of antihypertensive drug response

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We aimed to develop a microarray genotyping system for multiplex analysis of a panel of single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in blood pressure regulation, and to apply this system in a pilot study demonstrating its feasibility in the pharmacogenetics of hypertension. A panel of 74 SNPs in 25 genes involved in blood pressure regulation was selected from the SNP databases, and genotyped in DNA samples of 97 hypertensive patients. The patients had been randomized to double-blind treatment with either the angiotensin II type 1 receptor blocker irbesartan or the β_1-adrenergic receptor blocker atenolol. Genotyping was performed using a microarray based DNA polymerase assisted 'minisequencing' single nucleotide primer extension assay with fluorescence detection. The observed genotypes were related to the blood pressure reduction using stepwise multiple regression analysis. The allele frequencies of the selected SNPs were determined in the Swedish population. The established microarray-based genotyping system was validated and allowed unequivocal multiplex genotyping of the panel of 74 SNPs in every patient. Almost 7200 SNP genotypes were generated in the study. Profiles of four or five SNP-genotypes that may be useful as predictors of blood pressure reduction after antihypertensive treatment were identified. Our results highlight the potential of microarray-based technology for SNP genotyping in pharmacogenetics. *Pharmacogenetics* 13:7–17 © 2003 Lippincott Williams & Wilkins

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Introduction

The most abundant form of genetic variation are the single nucleotide polymorphisms (SNPs) that occur on the average at one out of every thousand bases in the human genome [1,2]. As a result of the efforts of the SNP consortium, which comprises a collaboration between 14 major pharmaceutical companies, and the Human Genome Project [1], there are over four million SNPs in public databases. Depending on where in the genome a SNP occurs, it may have different consequences on the phenotypic level. SNPs in the coding regions of genes may alter the function or the structure of the encoded proteins. While most of the SNPs are located in non-coding regions of the genome and have no direct known impact on the phenotype of an individual, they are useful as genetic markers because they may be inherited linked to the functional variants of physically closely located genes.

Hypertension is a quantitative trait caused by multiple factors that interact through pathways involving cardiac function, blood volume, salt regulation, peripheral vascular tone and endothelial function [3]. Many of the components of the blood pressure regulating pathways are proteins that vary in structure and activity among individuals owing to SNPs in the genes encoding them. Twin studies show that heritable components explain approximately one-half of the variance in blood pressure [4,5]. Because genetic factors contribute to hypertension, there is good reason to hypothesize that genetic factors also contribute to individual responses to treatment with antihypertensive drugs. A large inter-individual variation in the response to antihypertensive treatment is well documented [6,7]. Despite attempts to use biochemical indicators, such as plasma renin activity, or metabolic characteristics as predictors of individual drug response, no such predictor has yet been identified to be of clinical importance [8,9].

We have recently found that the insertion/deletion polymorphism in the angiotensin-converting enzyme (ACE) gene predicted the reduction in blood pressure

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in hypertensive patients treated with an angiotensin II type 1 (AT_1)-receptor blocker, but not in patients treated with a β_1-adrenergic receptor (β_1-AR) blocker [10]. A SNP in the aldosterone synthase gene (CYP11B2 −344 T/C) has also been found to be predictive of the systolic blood pressure reduction in hypertensive patients treated with irbesartan [11]. These studies suggest that pharmacogenetic analyses could be used as tools to achieve the optimal therapy. The unimodal frequency distribution of the response to treatment with antihypertensive drugs on the population level shows that many factors are involved [7]. In this study, we hypothesized that a combination of SNPs in genes known to be involved in blood pressure regulation would be powerful for predicting individual responses to antihypertensive drug treatment.

To facilitate simultaneous analysis of multiple SNPs in blood pressure-related genes, we developed a microarray-based genotyping system that utilizes 'minisequencing' single nucleotide primer extension [12]. This reaction principle has been adapted to a variety of assay formats [13], and allows specific genotyping of most SNPs at the same reaction conditions, which is a particularly advantageous feature for multiplex genotyping in the microarray format. In our system, the primers are immobilized covalently on microscope slides in an 'array of arrays' format [14] that allows genotyping of a panel of SNPs in multiple samples on each slide. We applied the microarray-based minisequencing system to genotype a panel of 74 SNPs in blood pressure regulating candidate genes in DNA samples from hypertensive patients who had been treated with either of two antihypertensive drugs with different mechanisms of action.

Materials and methods

Study populations and blood samples

DNA samples from 97 hypertensive patients from the double-blind parallel group 'Swedish Irbesartan Left Ventricular Hypertrophy Investigation versus Atenolol' (SILVHIA) trial [15] were analysed. The patients were randomized to treatment with either an AT_1-receptor blocker (irbesartan) or a β_1-AR blocker (atenolol). The results presented in this study are reported after 3 months of monotherapy. In 66% of the subjects receiving irbesartan and 39% of the subjects receiving atenolol, the dose was doubled after 6 weeks of treatment. However, neither for irbesartan nor for atenolol were the doses significantly related to the change in blood pressure (data not shown). The reduction in seated systolic blood pressure (SBP) and diastolic blood pressure (DBP) was recorded in a strictly standardized manner, as previously described in detail [10,15]. The two treatment groups showed a similar reduction in SBP and DBP. Table 1 presents the basic characteristics of the patients.

Table 1. Basic characteristics of the hypertensive patients

	Irbesartan group*	Atenolol group*	*P*
Number of patients	48	49	
Age (years)	54 ± 8	54 ± 8	0.94
Gender (proportion females)	37%	31%	0.36
Height (m)	1.74 ± 0.09	1.73 ± 0.09	0.61
Weight (kg)	83 ± 15	82 ± 14	0.73
Pretreatment SBP (mmHg)	164 ± 18	160 ± 20	0.40
Pretreatment DBP (mmHg)	104 ± 7	103 ± 8	0.43
Change in SBP at 12 weeks (%)	−9.9 ± 11	−6.8 ± 9.3	0.13
Change in DBP at 12 weeks (%)	−9.6 ± 9.7	−11 ± 7.0	0.34

*Data are mean ± SD.

The study was approved by the ethics committee of the Medical Faculty of Uppsala University (DNr 01–378).

A pooled sample consisting of equal amounts of DNA from 150 healthy Swedish blood donors was analysed to validate the SNPs and to determine the population frequencies of the SNP alleles. DNA was extracted from the blood samples using QiAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) or the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA).

SNPs and primers

Ninety-eight SNPs in candidate genes were identified from the literature and the dbSNP (www.ncbi.nlm.nih.gov/SNP) and TSC (The SNP consortium, http://snp.cshl.org/) databases. They are described in greater detail in Table 2. The polymerase chain reaction (PCR) primers were designed using the Primer Analysis Software Oligo, version 6.15 (Molecular Biology Insights Inc, Cascade, Colorado, USA) to have similar T_m values, two or three A- and/or C-residues at their 3′-ends and universal primer sequences at their 5′-ends. The primers were synthesized by Interactiva Biotechnologie GmbH (Ulm, Germany) or by Sigma-Genosys (Cambridge, UK). The sequences of the primers are available from the authors upon request.

PCR

PCR primers for 49 fragments comprising 74 SNPs were combined into eight multiplex PCR reactions containing two to 10 primer pairs, generating PCR products of different sizes. The fragments were amplified using a touchdown PCR-program [16] in a Thermal Cycler PTC-225 (MJ Research, Watertown, Massachusetts, USA). The PCR reactions were performed with 50 ng of DNA, 4 U of AmpliTaq Gold DNA polymerase (N808–0245, Applied Biosystems, Foster City, California, USA), 3.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 0.2–0.5 μmol/l primer and 5% dimethylsulphoxide in

Table 2. **Description of the single nucleotide polymorphisms analysed**

Gene name and acronym	Accession number[a]	SNP name[b,c]	dbSNP ID[d]	Amino acid
Renin–angiotensin–aldosterone system				
Aldosterone synthase	D13752	CYP11B2 T267C (344T/C)	rs1799998	
CYP11B2	–	CYP11B2 A3323G	NA	Lys/Arg
Angiotensin converting enzyme	AF118569	ACE C10514T	rs4316	
ACE	–	ACE T10527C	rs4317	Ser/Ala
	–	ACE A10578G	rs4318	Ser/Gly
	–	ACE G12257A	rs4331	
	–	ACE G14480C	rs4341	
	–	ACE C14488A	rs4342	
	–	ACE A14521G	rs4343	Thr/Thr
Angiotensinogen	M24686	AGT C1015T (T174M)	rs4762	Thr/Met
AGT	–	AGT T1198C (M235T)	rs699	Met/Thr
	–	AGT A1237G	NA	Tyr/Cys
	X15323	AGT A1204C (A-20C)	rs5050	
	–	AGT G1218A (G–6A)	rs5051	
Angiotensin II type 1 receptor	AF245699	AGTR1 A49954G	rs5183	Pro/Pro
AGTR1	–	AGTR1 A50058C (A1166C)	rs5186	
	–	AGTR1 T4955A	rs275651	
	–	AGTR1 T5052G	rs275652	
	–	AGTR1 C5245T	rs1492078	
Angiotensin II type 2 receptor	U20860	AGTR2 G1675A	rs1403543	
AGTR2	–	AGTR2 G4297T	rs5193	
	–	AGTR2 A4303G	rs5194	
	U20860	(AGTR2 T3786C)	rs5192	Ala/Ala
Mineralocorticoid receptor	M16801	MLR C1825T	NA	Arg/Term
MLR				
Renin	M26900	REN T1456G	rs5707	
REN	–	REN A1442G	rs5706	
	M26440	REN G134A	NA	
	–	REN T164G	NA	
	M26900	REN A279C (A204C)	rs5705	Thr/Thr
	L00073	(REN C170T)	NA	Arg/Term
Adrenergic system				
Adrenergic α_{1a} receptor	NM_000680	ADRA1a T1441C	NA	Arg/Cys
ADRA1a				
Adrenergic α_{1b} receptor	NM_000679	(ADRA1b G1234C)	NA	Gly/Arg
ADRA1b				
Adrenergic α_2 receptor	M23533	ADRA2A C787G	rs1800544	
ADRA2A	–	ADRA2A G1817A	rs1800545	
	–	ADRA2A G278T	rs1800763	
Adrenergic β_1 receptor	AF169006	ADRB1 C1165G	rs1801253	Arg/Gly
ADRB1	–	ADRB1 A145G	rs1801252	Gly/Ser
Adrenergic β_2 receptor	J02960	ADRB2 T1217C	rs1042711	Arg/Cys
ADRB2	–	ADRB2 T1244C	rs1801704	
	–	ADRB2 G1309A (G46A)	rs1042713	Arg/Gly
	–	ADRB2 G1342C (G79C)	rs1042714	Glu/Gln
	–	(ADRB2 T1754C (Ile164))	rs1800888	Thr/Ile
Adrenergic β_3 receptor	X72861	ADRB3 T827C	rs4994	Trp/Arg
ADRB3	–	ADRB3 C3246T	rs4999	
Endothelial system				
Endothelin receptor type A	D11149	EDNRA T89C	rs5333	His/His
EDNRA	–	EDNRA G125A	rs5334	Glu/Glu
Endothelin receptor type B	D13165	EDNRB G40A	rs5351	Leu/Leu
EDNRB				
Endothelial nitric oxide synthase	D26607	eNOS G7002T	rs1799983	Asp/Glu
ENOS	AF032908	eNOS A2000T	rs1800783	
	D26607	eNOS G9767A	rs1800780	
	–	eNOS A498G	rs1800779	
	–	eNOS G20455T	rs1065299	
	–	eNOS G2996A	rs1800781	
Lipid metabolism				
Apolipoprotein A	J02758	APOA A1449G	rs5092	Thr/Thr
APOA				
Apolipoprotein B	M19828	APOB G10108A	rs1801701	Arg/Gln
APOB	M19810	APOB C711T	rs1367117	Thr/Ile
Apolipoprotein E	M10065	APOE T3932C (T112C)	rs429358	Arg/Cys
APOE	–	APOE C4070T (C158T)	rs7412	Arg/Cys
Cholesteryl ester transfer protein	U85248	CETP A128C	NA	
CETP	–	CETP C146A	rs1800776	
	–	CETP C147A	rs1799851	
	M32997	CETP A1300G	rs5882	Ile/Val
	–	CETP G1335A	rs5886	Val/Val

(continued)

Table 2. *(continued)*

Gene name and acronym	Accession number[a]	SNP name[b,c]	dbSNP ID[d]	Amino acid
	M32998	CETP G338A	rs1800777	Arg/Gln
	–	CETP G571A	rs5887	Val/Met
	–	CETP A311G	rs2303790	Asp/Gly
	M32993	(CETP G2132T)	NA	Gly/Term
	M32992	(CETP T1511G)	NA	Tyr/Term
Low density lipoprotein receptor	AF217403	LDLR C16730T	rs688	Asn/Asn
LDLR	–	LDLR T20001C	rs5925	Val/Val
Lipase hepatic	M35429	LIPC G89A	NA	Arg/His
LIPC	–	LIPC A110G	rs6083	Asn/Ser
	J03895	(LIPC C873T)	NA	Ser/Phe
	–	(LIPC A1075C)	NA	Leu/Phe
	–	(LIPC C1221T)	NA	Thr/Met
Lipoprotein lipase	AF050163	LPL C9040G	rs328	Ser/Term
LPL	–	LPL G7315C	rs312	
	–	LPL A7344G	rs313	
	–	LPL G7360A	rs314	
Other				
Calcium sensing receptor	X81086	CASR C3031G	rs1801726	Gln/Glu
CASR	–	CASR G2956T	rs1801725	Ala/Ser
	–	(CASR G2968A)	rs1042636	Gly/Arg
Endothelin 2	M65199	(ET2 T461C)	rs5798	Phe/Leu
ET2		(ET2 G577A)	rs5799	Arg/Arg
Glucocorticoid receptor	M69104	(GRL A1502G)	rs6195	Asn/Ser
GRL				
Lecithin-cholesterol acyltransferase	X04981	(LCAT G2233A)	NA	Arg/His
LCAT				
Neuropeptide Y	K01911	(NPY T106C)	rs16139	Leu/Pro
NPY				
Osteoprotegerin	AB008821	(OPG T950C)	rs2073617	
OPG	–	(OPG C1217T)	NA	
	–	(OPG G1181C)	rs2073618	Lys/Asn
Parathyroid hormone	J00301	(PTH T164C)	NA	Cys/Arg
PTH	–	(PTH T179C)	NA	Ser/Pro
Peroxisome proliferator activated receptor α	AL078611	PPARA T39067C	rs1800234	Val/Ala
PPARA				
Peroxisome proliferator activated	L40904	PPARG G1043A	NA	Val/Met
receptor gamma	–	PPARG C1575T	NA	Pro/Leu
PPARG				
Superoxide dismutase	L44135	(SOD C386T)	NA	Ala/Val
SOD	L34157	(SOD C2734T)	rs1799725	Ala/Val
	U10116	(SOD C5775G)	NA	Arg/Gly
Vitamin D receptor	AC004466	(VDR T12022C)	NA	Met/Thr
VDR				

[a]Accession number of GenBank entry. [b]SNP name composed of acronym, nucleotide variation and nucleotide position. Alternative SNP name in parentheses. [c]SNPs marked with parentheses were excluded from genotyping panel. [d]dbSNP ID number; NA, not available.

100 µl of PCR reaction buffer supplied with the enzyme. The multiplex PCR reactions had been optimized with respect to $MgCl_2$, enzyme and primer concentrations. The success of the PCR was evaluated on 8% polyacrylamide gels.

Preparation of microarrays

The minisequencing primers were covalently immobilized on CodeLink™ Activated slides (previously 3D-Link slides, Motorola, Northbrook, Illinois, USA) by mediation of a 5′-NH_2 group [17]. The NH_2-modified primers were applied to the slides at a 25 µmol/l concentration in 400 mmol/l sodium bicarbonate buffer, pH 9.0, by contact printing using a ProSys 5510A spotter (Cartesian Technologies Inc., Irvine, California, USA) equipped with four Stealth Micro Spotting Pins (SMP3; TeleChem International Inc., Sunnyvale, California, USA) resulting in spots with a diameter of 160 µm. The oligonucleotides were printed in duplicate spots with a centre-to-centre distance of 200 µm in an 'array of arrays' format with the same spacing as the wells of a 384-well microtitre plate [14] with four columns and 14 rows on each slide. A fluorescently labelled oligonucleotide was included as control for the immobilization process and for scanning of the arrays.

Microarray minisequencing reactions

The multiplex PCR products from each sample were combined, precipitated with ethanol and the DNA pellet was resuspended in 46 µl of water. After denaturation of the DNA at 95 °C for 2 min, 14 µl of a buffer containing 4.5 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 8.0, 5 mmol/l Na_2-EDTA was added, and 15 µl of the DNA solution was used in four parallel

reaction wells on the slides. A custom made silicon rubber grid placed on top of each microscope slide was used to form 56 separate reaction chambers [14]. A heated aluminium block holding three microscope slides was used for the reactions. After incubation for 40 min at 37 °C, the slides were briefly rinsed in a solution containing 5 mmol/l Tris-HCl pH 8.0, 0.5 mmol/l Na_2-EDTA, 100 mmol/l NaCl, 0.1% Triton X-100 followed by rinsing with water. The minisequencing reaction mixtures contained one of the four TAMRA-labelled ddNTPs (ddATP NEL474, ddCTP NEL473, ddGTP NEL475 or ddUTP NEL472 Perkin-Elmer Life Sciences, Boston, Massachusetts, USA) and the other three ddNTPs unlabelled at a concentration of 0.5 μmol, 0.75 U of DynaSeq DNA polymerase (gift from Finnzymes OY, Helsinki, Finland) or Thermo Sequenase DNA Polymerase (Amersham Biosciences, Uppsala, Sweden) in 26 mmol/l Tris-HCl pH 9.5, 6.5 mmol/l $MgCl_2$, 0.1% Triton X-100. Fifteen μl of reaction mixture was added to the reaction wells on the slides preheated to 55 °C, followed by incubation for 15 min. The slides were then briefly rinsed in water followed by washing for 2 min with 50 mmol/l NaOH, and twice for 5 min with 3 mmol/l sodium citrate, 30 mmol/l NaCl, 0.1% SDS, pH 7.0 at 65 °C and finally briefly rinsed with water. The slides were allowed to dry at room temperature before fluorescence scanning.

Fluorescence scanning and data interpretation

The fluorescence signals were detected using an array scanner (ScanArray 5000, Perkin-Elmer Life Sciences, Boston, Massachusetts, USA). The fluorescence intensity values were extracted with the QuantArray software supplied with the scanner. Using an algorithm, the mean values of the signals from the duplicate spots were corrected for the average background measured beneath each 'subarray'. The ratio between the fluorescence signal from one of the alleles and the sum of the fluorescence signals from both possible alleles at each SNP site was calculated to assign the genotypes.

Minisequencing in microtitre plates

Solid-phase minisequencing in microtitre plates with ^{3}H-labelled dNTPs was used to determine the allele frequencies of the SNPs in pooled DNA samples essentially as described previously [12,18], with the exception that a biotin residue for immobilization of the PCR products in the streptavidin-coated microtitre well was incorporated in a secondary PCR reaction with a biotinylated primer complementary to the universal sequence on the specific PCR primer. The ratio between the ^{3}H-dNTPs incorporated was normalized against the ratio in a heterozygous sample. If no heterozygous reference sample was available, the specific activities of the tritium labelled nucleotides (^{3}H-dATP TRK633 69 Ci/mmol, ^{3}H-dCTP TRK625 46 Ci/mmol, ^{3}H-dGTP TRK627 41 Ci/mmol, ^{3}H-dTTP TRK576 124 Ci/mmol, Amersham Biosciences) were used for normalization. Solid-phase minisequencing also served as reference method [12].

Statistical analysis

For the SNPs with a frequency over 5% for the minor allele, Hardy–Weinberg equilibrium was assessed by comparison of the observed and expected genotype frequencies using the chi-square test with one degree of freedom. The Genepop web program (http://wbiomed.curtin.edu.au/genepop) [19] was used to calculate pairwise linkage disequilibrium (LD). Haplotypes were designated using the expectation maximization algorithm in the Haplo program (http://info.med.yale.edu/genetics/kkidd) [20]. Subsets of SNPs required for distinguishing between haplotypes [21] were identified by visual inspection of the haplotypes.

The relationship between genotypes and change in blood pressure was first analysed using factorial one-way ANOVA (Statview 5, SAS Institute Inc., Cary, North Carolina, USA) to evaluate which of the genotypes of the 74 SNPs were related to the changes in SBP or DBP in the irbesartan and atenolol group separately. Four univariate analyses were performed for each of the individual SNPs. SNPs demonstrating a $P < 0.10$ were entered as independent variables in forward stepwise multiple regression models with one of the four possible combinations of SBP/DBP and irbesartan/atenolol as a dependent variable. In these models, $P < 0.01$ was set for retention of the independent variables in the models in order to reduce the effect of multiple testing by reducing the P-value accepted. Two-tailed significance levels were used. No formal correction for multiple testing was applied.

Results

The candidate genes were chosen based on their function in the renin–angiotensin–aldosterone, adrenergic and endothelial systems, and the lipid metabolism, respectively. A panel of 98 genetic variants in these genes was assembled for prediction of individual responses to treatment with antihypertensive drugs (Table 2). This panel comprised a small number of SNPs that had been shown or suggested to be involved in blood pressure regulation in the published literature. Most of the SNPs in the panel were located in coding or regulatory regions of the candidate genes, but also a few SNPs in non-coding regions close to the genes (< 1.5 kb) were included. When possible, SNPs with published allele frequencies from 10% to 90% were selected. The allele frequencies in the Swedish population of the initially selected SNPs were determined by quantitative minisequencing analysis [12,18] of a pooled sample containing DNA from 150 healthy individuals of Swedish origin. Based on this analysis, 24 SNPs were excluded from the panel for SNP profiling

because they had a minor allele frequency of less than 1% in the Swedish population (16 SNPs) or due to PCR failure (8 SNPs). Over one-half of the SNPs, especially those with even allele distribution, had similar frequencies in Sweden as in the population where they had been identified (Table 3). Seventy-four SNPs with allele frequencies ranging from 1% to 50% for their minor allele were included in the panel to genotype the DNA samples from hypertensive patients.

Figure 1 presents a fluorescence scan image of a microarray where the SNPs included in the panel have been genotyped by minisequencing in samples from 12 of the patients. After fluorescence scanning of the arrays, the results are interpreted with the aid of an algorithm that extracts the signals from the microarray, and assigns the genotypes according to the ratio of the fluorescence signal from one of the alleles to the sum of the fluorescence signals from both alleles at each SNP site. As shown in Fig. 2, the signal ratios for each SNP fall within one of three distinct, non-overlapping clusters that define the three possible genotypes unequivocally. Although the limits for the clusters of ratios vary depending on the sequence context of the SNPs, the ratios defining the genotype of the SNPs differ from each other on average by 0.32.

Using the microarray-based system, the panel of SNPs was genotyped in DNA samples from the 97 hypertensive patients participating in the study. As a consequence of the stringent criteria applied for defining the genotypes, approximately 20% of the genotypes were assigned after repeating the microarray-based analysis with a primer of the opposite polarity, or by verification of individual genotypes in the microtitre plate format of the minisequencing method. As a result of this strategy, a genotype was assigned for each SNP in every patient, and thus almost 7200 genotypes were generated. The genotypes of four SNPs (CYP11B2 T267C, AGTR1 A50058C, ADRB1 A145G, ADRB2 T1217C) were also determined individually by the reference method in all of the study samples. In addition, 200 randomly selected genotypes (8%) were redetermined by the reference method. Fully concordant genotyping results were obtained by the individual analysis and by the multiplex microarray-based genotyping, evidencing for the accuracy of the microarray system. The assigned genotypes conformed to Hardy–Weinberg expectations with the exception of the three SNPs in the AT_2 receptor gene, the C1165G SNP in the β_1-adrenergic receptor gene and the G134A SNP in the renin gene ($P < 0.001$).

The allele frequencies for the panel of SNPs calculated from the individual patient samples are shown in Table 3. With the exception of the SNPs REN G134A,

Table 3. Allele frequency of the single nucleotide polymorphisms included in the panel

SNP	Allele frequency		
	Literature[a]	Sweden	Study subjects
Renin–angiotensin–aldosterone system			
CYP11B2 T267C	57/43	61/39	61/39
ACE C10514T	59/41	63/37	58/42
ACE T10527C	86/14	94/6	98/2
ACE A10578G	86/14	98/2	99/1
ACE G12257A	41/59	52/48	54/46
ACE G14480C	59/41	61/39	53/47
ACE C14488A	41/59	54/46	52/48
ACE A14521G	59/41	52/48	47/53
AGT C1015T	88/12	87/13	87/13
AGT T1198C	59/41	52/48	54/46
AGT A1237G	NA	99/1	99/1
AGT A1204C	90/10	85/15	84/16
AGT G1218A	30/70	65/35	57/43
AGTR1 A49954G	75/25*	95/5	97/3
AGTR1 A50058C	50/50*	74/26	77/23
AGTR1 T4955A	82/18	70/30	73/27
AGTR1 T5052G	83/17	77/23	79/21
AGTR1 C5245T	64/36	70/30	47/53
AGTR2 G1675A	56/44	51/49	49/51[b]
AGTR2 G4297T	60/40	85/15	77/23[b]
AGTR2 A4303G	30/70	58/42	49/51[b]
MLR C1825T	NA	99/1	99/1
REN T1456G	55/45*	71/29	74/26
REN A1442G	95/5*	99/1	98/2
REN G134A	NA	85/15	31/69[b]
REN T164G	NA	83/17	93/7
REN A279C	65/35*	84/16	88/12
Adrenergic system			
ADRA1A T1441C	NA	92/8	ND
ADRA2A C787G	73/27	82/18	76/24
ADRA2A G1817A	89/11	82/18	89/11
ADRA2A G278T	95/5	98/2	96/4
ADRB1 C1165G	74/26	71/29	38/62[b]
ADRB1 A145G	87/13	72/28	72/28
ADRB2 T1217C	57/43	63/37	57/43
ADRB2 T1244C	NA	60/40	64/36
ADRB2 G1309A	63/37	63/37	54/46
ADRB2 G1342C	66/34	60/40	37/63
ADRB3 T827C	85/15	87/13	91/9
ADRB3 C3246T	80/20*	98/2	98/2
Endothelial system			
EDNRA T89C	74/26	82/18	75/25
EDNRA G125A	74/26	63/37	68/32
EDNRB G40A	75/25	72/28	70/30
ENOS G7002T	61/39	64/36	77/23
ENOS A2000T	45/55	62/38	ND
ENOS G9767A	52/48	79/21	72/28
ENOS A498G	56/44	65/35	71/29
ENOS G20455T	68/32	59/41	62/38
ENOS G2996A	85/15	87/13	73/27
Lipid metabolism			
APOA A1449G	65/35	68/32	74/26
APOB G10108A	90/10	84/16	87/13
APOB C711T	68/32	78/22	67/33
APOE T3932C	86/14	78/22	81/19
APOE C4070T	93/7	91/9	93/7
CETP A128C	53/47	99/1	100/0
CETP C146A	92/8	92/8	97/3
CETP C147A	63/37	99/1	100/0
CETP A1300G	68/32	72/28	64/36
CETP G1335A	97/3	95/5	99/1
CETP G338A	97/3	91/9	ND
CETP G571A	95/5	97/3	100/0
CETP A311G	NA	97/3	99/1
LDLR C16730T	67/33	56/44	52/48
LDLR T20001C	67/33	55/45	54/46
LIPC G89A	NA	99/1	99/1
LIPC A110G	33/67	57/43	65/35

(continued)

Table 3. *(continued)*

SNP	Allele frequency		
	Literature[a]	Sweden	Study subjects
LPL C9040G	88/12	90/10	93/7
LPL G7315C	75/25	86/14	81/19
LPL A7344G	94/6	91/9	100/0
LPL G7360A	61/39	95/5	64/36
Other			
CASR C3031G	95/5	82/18	93/7
CASR G2956T	84/16	94/6	90/10
PPARA T39067C	NA	99/1	100/0
PPARG G1043A	NA	99/1	100/0
PPARG C1575T	NA	99/1	100/0

[a]Allele frequency in a European population except for a few from Zimbabwe marked with an asterisk. NA, frequency not available. [b]Deviation from Hardy–Weinberg equilibrium, $P < 0.001$.

ADRB1 C1165G, ADRB2 G1342C and LPL G7360A, the allele frequencies identified in the hypertensive patients were identical to those in the pooled sample representing healthy blood donors.

The most probable haplotypes were determined using the expectation maximization algorithm based on the genotypes of the SNPs in eight of the candidate genes (Fig. 3). This analysis revealed that the allelic diversity of these genes is explained by three to six major haplotypes with a frequency of more than 5%. This analysis also shows that only two or three SNPs per gene define the haplotypes in 73% to 95% of the individuals analysed.

Of the 97 individuals with hypertension included in the study, one-half had been treated with atenolol and half with irbesartan. There was no difference in the basic characteristics between the two treatment groups, and both groups showed a similar reduction in SBP and DBP at three months of treatment (Table 1). Because the reduction in blood pressure was unrelated to the doses of the two drugs, the doses were not evaluated as confounders in the statistical analysis. Those SNPs that were related to a change in blood pressure by univariate analysis were subjected to stepwise multiple regression analyses relating the genotypes of the SNPs to changes in blood pressure. This analysis revealed profiles of four or five SNPs that appeared as significant predictors of the reduction in SBP and DBP (Table 4).

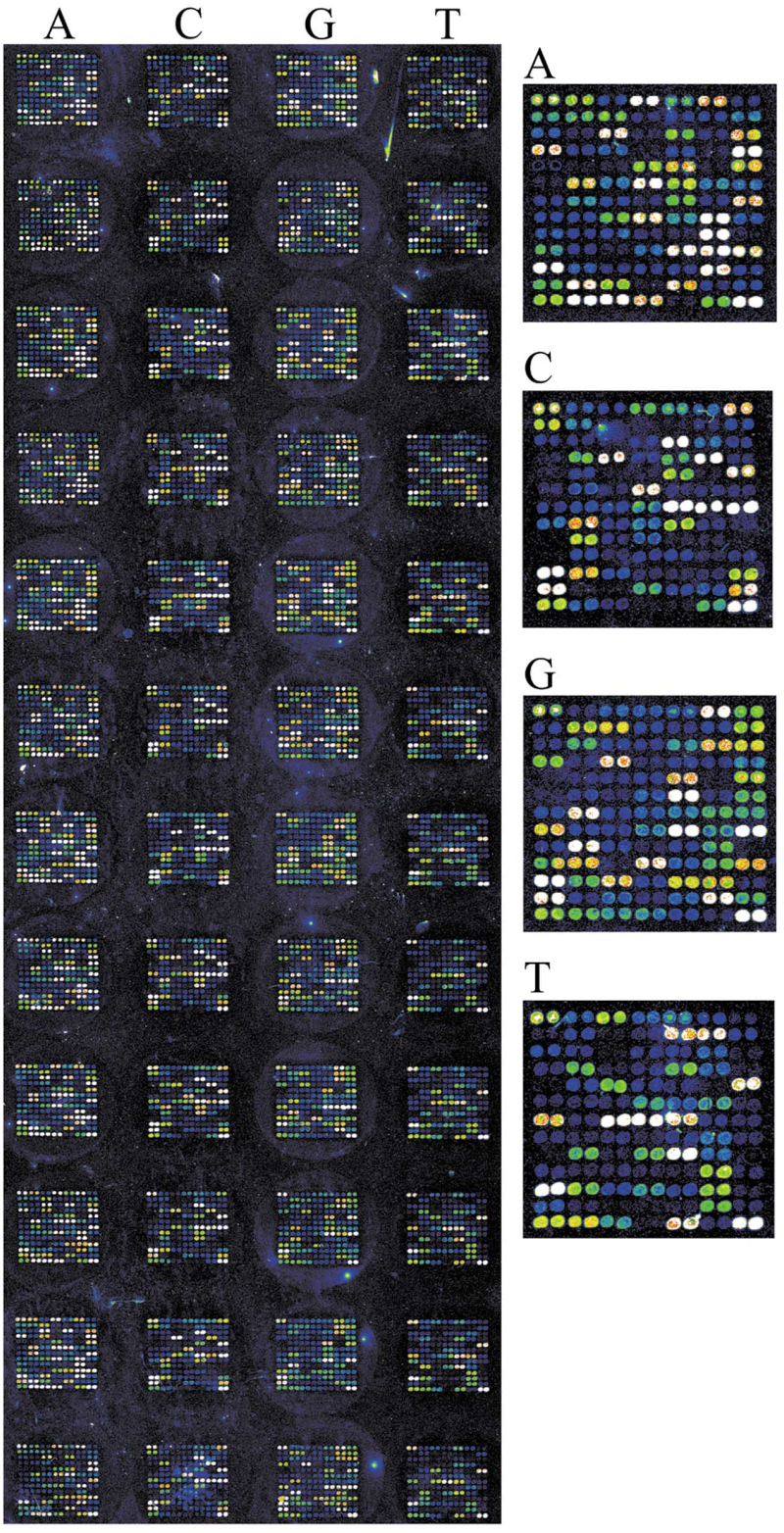
According to the calculation of pairwise linkage disequilibrium by the Genepop program, the two SNPs in the adrenergic receptor α_2 in the group treated with atenolol, as well as the two SNPs in the ADRB2-gene that predicted both the SBP and DBP responses, are in LD, respectively. In the irbesartan-treated group, SNPs in the genes encoding angiotensinogen, ACE and aldosterone synthase were significant predictors of blood pressure reduction. The SNP G12257A in the ACE gene is in complete LD with the ACE I/D polymorphism, which was shown to predict the reduction in blood pressure in the same patients treated with irbesartan in our previous study [10]. In both treatment groups, the SNPs in the endothelin type B receptor (G40A) and the endothelial nitric oxide synthase (eNOS) (A498G) genes were predictors of SBP reduction. Of the SNPs in genes encoding components of lipid metabolism, the same SNP in the apolipoprotein A gene (A1449G) emerged as predictor of both SBP and DBP response to irbesartan. The SNP in the hepatic lipase gene (A110G) showed opposite effects on DBP response in the irbesartan and atenolol groups. In both treatment groups, the combined SNP genotype profiles explain as much as 44% to 56% of the reduction in SBP and DBP.

Discussion

In this study, we have established a robust microarray-based minisequencing system for genotyping a panel of 74 SNPs in 25 genes encoding proteins that potentially determine the pharmacodynamics of individual responses to antihypertensive treatment. The system proved to be robust and allowed accurate genotyping of all SNPs in every sample. Using this genotyping system, we were able to identify combinations of a few SNPs that predicted approximately 50% of the variation in the individual drug responses in two groups of hypertensive patients that had been subjected to monotherapy with one of two different antihypertensive drugs, a β_1-AR blocker or an AT_1-receptor blocker. The number of subjects in our study was low, and the results discussed below should be considered as hypothesis-generating and need to be confirmed in a larger sample set.

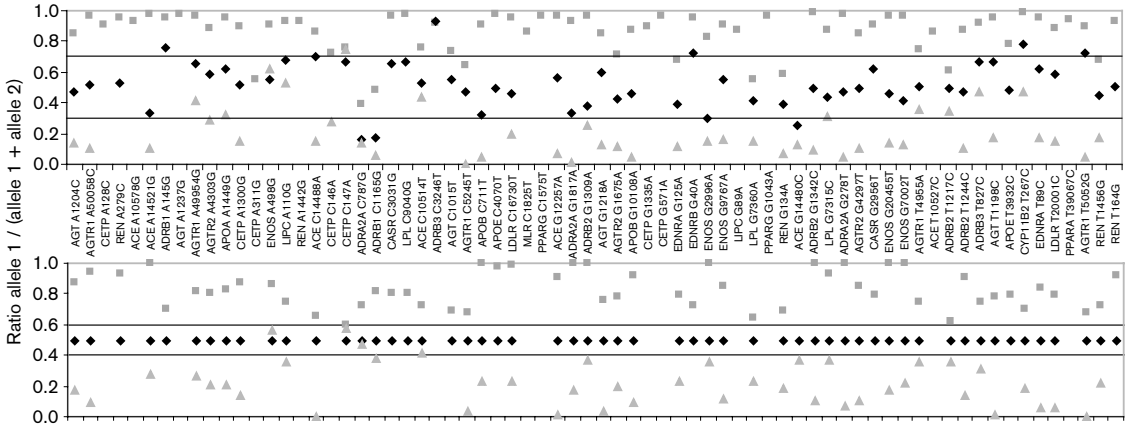
Certain features of the combinations of genetic variants that we identified as predictors of antihypertensive response are of particular interest. First, none of the investigated SNPs in the genes encoding the β_1-AR or the AT_1-receptor, which are the targets for irbesartan and atenolol, respectively, were useful predictors. The reasons for this could be that a relevant SNP was not included in the panel due to allelic heterogeneity of the genes, or that other steps in the physiological chains involved in the action of a drug are more important. The latter assumption might be supported by the fact that SNPs in the aldosterone synthase, ACE and angiotensinogen genes, which are all important components in the renin–angiotensin–aldosterone system, were among the genetic variants that were predictive for the response to the AT_1-receptor blocker. Similarly, SNPs in the β_2-adrenergic and adrenergic α_2 receptors were predictors for the response to β-blockade. Second, both the endothelin type B receptor and the eNOS genes were found in the combinations that predicted the SBP response to both drugs, suggesting that an effect mediated by the endothelium is a common

Fig. 1



Fluorescence image of a minisequencing microarray. Twelve samples have been genotyped for the panel of 74 SNPs. Each microscope slide carries 56 individual 'subarrays'. Primers immobilized in the 'subarrays' have been extended with the combined PCR amplified DNA fragments spanning the SNPs as templates with TAMRA-labelled nucleotide analogues in four separate minisequencing reactions using a DNA polymerase. Four 'subarrays' are shown enlarged. The rainbow colour scale corresponds to different signal intensities with blue as low and white as saturated signal.

Fig. 2



Power of SNP genotype discrimination of the microarray-based minisequencing system. Upper panel: average signal intensity ratios obtained for the panel of SNPs in 38 hypertensive patients in three experiments on separate microarrays. The limits of the clusters defining the genotypes were > 0.8 or < 0.2 for homozygous genotypes, and between 0.7 and 0.3 for the heterozygous for most of the SNPs. Alternatively, a difference of at least 0.1 in the ratios was applied to assign the genotypes. Lower panel: the average ratio for the heterozygotes in the upper panel has been normalized to 0.5, and the average ratios for the homozygotes plotted accordingly. Ratios for homozygotes falling below the theoretical value of 0 or above the value of 1 for homozygotes have been plotted as being 0 and 1, respectively. ■, homozygote common; ◆, heterozygote; ▲, homozygote rare.

action for both drugs. Third, although the SNPs APOA A1449G and EDNRB G40A predicted both the changes in SBP and DBP, most of the SNPs, found to be of interest predicted either SBP or DBP. This finding further emphasizes the fact that SBP and DBP are different entities, determined mainly by different mechanism, such as aortic stiffness and peripheral resistance. Finally, genes encoding components in lipid metabolism were found to be of predictive value for the blood pressure response to these antihypertensive drugs. This finding is in accordance with epidemiological studies showing a relationship between different lipid variables and blood pressure [22–24], which was the rational for including these genes in the analysis. Potentially interesting for future pharmacogenetic use is the finding that one SNP (A110G), causing an amino acid substitution in the hepatic lipase gene, predicted positive response to treatment with atenolol but not to treatment with irbesartan. Five patients had the 'favourable' genotype combination predicting response in SBP upon treatment with irbesartan. They showed a mean reduction in SBP of 45 mmHg, while the reduction of SBP in the other patients in this treatment group was as low as 11 mmHg. The complete 'favourable' genotype combination was present in only one or two patients from the other treatment groups.

Selection of the panel of SNPs for genotyping, and the allele frequency distribution for the SNPs in the Swedish population were obviously critical for the outcome of the study. Multiple SNPs in each of the candidate genes were included in the panel to account

Fig. 3

ACE

(C/T)	T/C	A/G	A/G	(G/C)	(A/C)	G/A	Freq. %
T	T	A	G	C	C	A	30
C	–	–	A	G	A	G	28
C	–	–	A	–	A	G	13
C	–	–	–	–	–	–	8.6

ADRB2

T/C	T/C	(G/A)	(C/G)	Freq. %
T	T	A	C	40
C	C	G	G	27
–	–	G	–	17
C	C	–	G	8.6

AGT

(C/T)	(T/C)	A/G	C/A	(A/G)	Freq. %
C	T	A	A	G	48
–	C	–	–	A	27
T	C	–	C	A	7.8

REN

(A/C)	(T/G)	A/G	(A/G)	G/T	Freq. %
A	T	A	A	T	39
–	–	–	G	–	17
–	G	–	–	–	13
C	–	–	–	–	8.9
–	G	–	G	–	8.3

CETP

A/C	(A/C)	(C/A)	(A/G)	G/A	G/A	A/G	Freq. %
A	A	C	A	G	G	A	31
–	–	–	G	–	–	–	21
–	C	–	–	–	–	–	19
–	C	–	G	–	–	–	8.9
–	–	A	–	–	–	–	8.9

LPL

C/G	(G/C)	(A/G)	Freq. %
C	G	G	61
–	–	A	15
–	C	A	12
G	–	A	6.9

ENOS

T/G	(G/A)	G/A	(T/G)	(A/G)	Freq. %
G	G	A	G	G	28
–	–	–	T	–	15
T	A	G	–	–	11
–	–	G	T	A	8.3
–	–	–	–	A	5.3
–	A	–	–	–	5.0

AGTR1

A/G	(A/C)	T/A	(T/G)	(C/T)	Freq. %
A	A	T	T	C	35
–	–	–	–	T	21
–	–	A	G	T	14
–	C	–	–	–	8.5
–	C	–	–	T	6.5

Haplotype frequencies for eight of the candidate genes for blood pressure regulation. Haplotypes with a frequency of $> 5\%$ are shown. The order of the SNPs in the haplotypes is the same as in Table 3. SNPs marked with a circle symbolize the smallest number of SNPs needed to distinguish the haplotypes in this set of samples. Dashes indicate the same nucleotide as in the most common haplotype.

Table 4. Stepwise regression models relating genotypes to the change in SBP and DBP during treatment with irbesartan or atenolol

SNP[a]	Genotype[b]	Regression coefficient[c]	*F*	*P*	Model r^2
SBP irbesartan					
APOA A1449G	AA	0.095	15.3	< 0.001	
CYP11B2 T267C	TT	−0.091	14.2	< 0.01	
EDNRB G40A	AA	0.102	10.5	< 0.01	
ENOS G498A	GG	0.109	7.3	< 0.01	0.51
SBP atenolol					
ADRA2A G278T	GG	−0.18	18.6	< 0.001	
ADRB2 G1342C	CC	−0.068	14.2	< 0.01	
AGT C1015T	CC	0.14	15.8	< 0.001	
EDNRB G40A	GG	−0.050	9.0	< 0.01	
ENOS G498A	AA	−0.074	19.4	< 0.001	0.56
DBP irbesartan					
APOA A1449G	AA	0.10	14.5	< 0.001	
ACE A12257G	GG	−0.073	9.7	< 0.01	
AGT C1198T	CC	0.10	8.4	< 0.01	
LIPC A110G	GG	−0.079	8.0	< 0.01	0.44
DBP atenolol					
ADRB2 G1309A	AA	0.053	8.4	< 0.01	
ENOS A2996G	AA	0.094	16.1	< 0.001	
ADRA2A G1817A	AA	−0.15	10.1	< 0.01	
LIPC A110G	GG	0.12	18.3	< 0.001	
EDNRB G40A	GG	−0.049	8.4	< 0.01	0.50

[a]See Table 2 for abbreviations of the SNPs. [b]Only genotypes with $P < 0.01$ were included. [c]A positive regression coefficient for a genotype should be regarded as a less pronounced reduction or, in some cases, even an increase in blood pressure compared to those carrying the other allele. Conversely, a negative regression coefficient indicate a more pronounced reduction in blood pressure compared to those carrying the other allele.

for allelic heterogeneity of the genes. However, it is notable that only five out of the 13 SNPs that constituted the profiles predicting SBP and DBP reduction, after antihypertensive treatment, encode an amino acid change in the corresponding protein, and could thus directly affect its biological function. The remaining predictor SNP variants are presumably in LD with functional variants not included in the analysis. According to our haplotype analysis of eight of the candidate genes containing multiple SNPs, three to six major haplotypes account for most of the allelic variation of these genes. The haplotypes describe the pattern of LD between adjacent SNPs and, if the haplotype structures had been known in advance, a smaller number of SNPs than those included in the genotyping panel would have been sufficient to capture the genetic variation of these eight genes.

Currently, large panels of SNPs located throughout the human genome are available in public databases. The assessment of the relationship between genetic variation and complex traits is hampered by the lack of high-throughput methods that would facilitate simultaneous genotyping of many SNPs in large sample sets; a review on SNP technology is provided elsewhere [25]. Homogeneous PCR assays with allele-specific probes or PCR primers, performed with real-time fluorescence detection [26,27] or primer extension assays in combination with mass spectrometric detection [28], have recently been the most favoured methods. Because these methods do not allow multiplex analysis of several SNPs per reaction, they are of limited use when large panels of SNPs are to be analysed in each sample. Microarray-based technology offers the potential of highly multiplexed assays, and DNA polymerase-assisted primer extension allows accurate SNP genotyping, even in low or intermediate density microarray formats. The standard microarray formats, originally designed for transcript profiling, are impractical for SNP typing in large sample sets because, typically, only one sample is analysed per microscope slide [29,30], which is the reason why these methods have not been widely applied beyond the 'proof of principle' level. The 'array of arrays' format employed in this study removed this obstacle, and allowed us to genotype multiple individual samples on each slide. The capacity of the genotyping system can be increased four-fold by using different fluorophores on each of the four nucleotides [31,32], instead of a single fluorophore, as was the case in this study. The major limitation in throughput of our method, as for all other SNP-genotyping methods, is imposed by the necessity of amplifying the regions spanning the SNPs by PCR. Provided that this problem can be solved, automation of the microarray-based assay would allow a genotyping throughput approaching that required for genotyping SNPs in all human genes, especially if a haplotype tagging strategy [21] were to be used for minimizing the number of SNPs required.

A limitation of our study is the small number of patients included. Another concern is the genotyping of multiple SNPs facilitated by the microarray-based genotyping system. Because this pilot study was mainly intended to establish and test the applicability of the genotyping system, and the results generated are exploratory, no formal adjustment for multiple testing was used. However, the stringent criteria of $P < 0.01$ was applied for inclusion of SNPs in the models in order to minimize the chance of type II errors. The issue of potentially false positive results due to multiple testing encountered in our study of 74 SNPs will be further accentuated in the future, when whole genome scans of thousands of SNP markers will become feasible owing to the development of high-throughput genotyping technology. It is obvious that traditional correction for multiple testing, such as Bonferoni correction, will not be applicable to this type of studies, because millions of study subjects would be required to achieve formal statistical significance. Hence, other solutions to the problem of multiple testing are needed. One solution would be to split the analysed sample population into two parts, and to use the second half of the samples to test the validity of the results obtained

in the first half of the samples. Unfortunately, this approach would have been possible in our pilot study only if a much larger number of samples had been available. Another, more fundamental solution would be to include samples from family members in the study, and to apply statistical genetics methods to interpret the genotyping data [33].

In conclusion, the preliminary results obtained in this pilot study need to be reproduced in a larger, prospective study to validate the identified pharmacogenetic profiles. We believe that, in the future, when a panel of predictive pharmacogenetic markers has been identified, multiplex genotyping assays such as the one designed here will be valuable as routine diagnostic tools for designing the optimal individualized drug treatment for hypertensive patients.

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