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ORIGINAL ARTICLE

The microsatellite alleles on chromosome 1 associated with essential hypertension and blood pressure levels

T Nakayama[1], M Soma[2], K Kanmatsuse[2] and S Kokubun[1]

[1]*Division of Receptor Biology, Advanced Medical Research Center, Nihon University School of Medicine, Ooyaguchi-kamimachi, Tokyo, Japan;* [2]*Second Department of Internal Medicine, Nihon University School of Medicine, Tokyo, Japan*

Essential hypertension (EH) is thought to be a polygenic disease. Several candidate genes of this disease have been investigated in studies using polymorphic genetic markers, but some studies have failed to show any association of EH with these genes. In this experiment, we used microsatellite markers on chromosome 1, and performed an association study between EH and control subjects. Genomic DNA was amplified with fluorescently labelled primers from the Applied Biosystems PRISM linkage mapping set HD-5 comprising 63 highly polymorphic microsatellite markers with an average spacing of 4.5 cM. We isolated three loci showing significant differences: D1S507, D1S2713 and D1S2842. The *P*-values of the allele with the greatest *post hoc* contributions in D1S507, D1S2713 and D1S2842 were 0.0008, 0.0062 and 0.0084, respectively. All these values were significant after Bonferroni correction. Furthermore, we found that the three microsatellite alleles were associated with the levels of systolic blood pressure. These data suggest that there are at least the three susceptibility loci for EH on chromosome 1, and that a case–control study using microsatellite markers on genomewide basis is a useful method for isolating the susceptibility loci of multifactorial disorders.

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Introduction

Essential hypertension (EH) is likely to be a polygenic disorder that results from the inheritance of a number of susceptibility genes. Although data from rodent models and human twin-based and population-based epidemiology studies suggest that inherited factors contribute 50% or less to the determination of an individual's eventual blood pressure (BP),[1] the number of contributing genes or their individual attributable risk remains unknown. Indeed, whether there are one or two major hypertension susceptibility-causing genes with several more minor loci or many genes, each with small attributable risks, is an important question that has not previously been possible to tackle. The affected sibling pair model has been used to identify loci in various complex traits by determining how often affected siblings share alleles.

During recent years, several scans of the human genome have been performed on families with multiple cases of EH. Many potential susceptibility loci have been identified.[2] Some genomewide scans have provided suggestive evidence of linkages on chromosome 1,[3–7] prompting detailed analysis of this region. Chromosome 1 includes genes of the natriuretic peptide system such as atrial natriuretic peptide,[8] brain natriuretic peptide, A-type natriuretic peptide receptor,[9–11] and genes of the renin–angiotensin system such as angiotensinogen[12] and renin.[13]

Genetic markers that are sufficiently polymorphic (as measured by their heterozygosities) can be used in linkage and association analyses to detect Mendelian segregation underlying disease phenotypes. Each type of analysis can either be based on a specific genetic model, or need not make any assumptions about the mode of inheritance of the disease. Association analyses are more powerful, provided there is linkage disequilibrium between the marker and the disease loci. Recently, it had been pointed out that when gametic disequilibrium is suspected, methods testing for both linkage and association might be more powerful.[14–15]

Correspondence: Dr T Nakayama, Division of Receptor Biology, Advanced Medical Research Center, Nihon University School of Medicine, Ooyaguchi-kamimachi, 30-1 Itabashi-ku, Tokyo 173-8610, Japan. E-mail: tnakayam@med.nihon-u.ac.jp
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The aim of the present study is to identify susceptibility loci of EH on chromosome 1 using microsatellite markers by a case–control study in Japanese subjects.

Materials and methods

Subjects

The EH group consisted of 144 patients with EH diagnosed according to the following criteria: sitting systolic blood pressure (SBP) greater than 160 mmHg and/or diastolic blood pressure (DBP) greater than 100 mmHg on three occasions within 2 months after the first medical examination.[10] Participants were not using antihypertensive medication. Subjects diagnosed with secondary hypertension were excluded. We also included 144 age-matched normotensive (NT) healthy subjects as controls. None of the NT subjects had a family history of hypertension, and they all had SBP less than 130 mmHg and DBP less than 85 mmHg. A family history of hypertension was defined as prior diagnosis of hypertension in grandparents, uncles, aunts, parents or siblings. Both the EH patients and the NT control subjects were recruited from the northern part of Tokyo, and informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee of Nihon University.

Biochemical analysis

Plasma concentrations of total cholesterol and HDL-cholesterol, and serum concentrations of creatinine and uric acid were measured as previously described.[16]

Polymerase chain reaction (PCR) and genotyping

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood mononuclear cells by standard procedures.[17] PCR was performed with fluorescently labelled primers from the Applied Biosystems PRISM linkage mapping set HD-5 comprising 63 highly polymorphic markers arranged in panels of compatible markers with an average spacing of 4.5 cM and average heterozygosity of 0.76. The primers were dye-conjugated with FAM, HEX or NED (Applied Biosystems). The loci were selected from the Genethon linkage map,[18] based on location on chromosome and heterozygosity. PCR amplification was carried out using 15 ng of genomic DNA, 3 μl True Allele PCR Premix (containing PCR buffer, $MgCl_2$, dNTP, Amplitaq Gold, Applied Biosystems) and 0.33 μl primer mix in a total reaction volume of 5 μl. Amplifications were carried out at 95°C for 12 min to activate the Amplitaq Gold; followed by 10 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 30 s; followed by 20 cycles of denaturation at 89°C for 15 s, annealing at 55°C for 15 s and extension at 72°C for 30 s; and ended with a final extension at 72°C for 10 min. Amplifications were run in a Gene Amp PCR 9600 thermocycler (Perkin Elmer, Foster City, CA, USA).

Products from up to eight different PCR reactions were pooled and diluted 1:10 for products labelled with FAM (1:5 for products labelled with HEX and NED). The pooled reactions (1 μl) were then mixed with 0.5 μl LIZ internal size standard GS-500 and diluted 1:6 with HI-DI formamide (Applied Biosystems). The pooled reactions were denaturated at 95°C for 3 min and were loaded on an ABI3700 DNA analyzer (Applied Biosystems). The fluorescent signal was recorded and analysed using the GeneScan version 2.1 software. Fragments from reactions using each of the different fluorescent dyes were plotted separately, and the sizes of fluorescent peaks were estimated for the base pairs by referencing the in-lane size standard. Marker alleles were classified according to their size using the Genotyper version 2 software. In addition to the automated allele calling, we performed manual surveillance of all genotypes.

Statistical analysis

Data represent mean $\pm$ s.d. Clinical data were tested using ANOVA followed by Fisher's protected least significant difference test, and *P*-values less than 0.05 indicated significant differences.

Hardy–Weinberg equilibrium was assessed by χ^2 analysis. The overall distribution of alleles between EH patients and NT subjects was analysed by $2 \times n$ contingency tables[19] and *P*-values less than 0.05 were considered significant. The calculations of expected values and *post hoc* cell contributions were also performed. Cells with expected values below 1 were omitted, and the frequency of expected values below 4 was set to below 20%.[20]

Individual differences in allele frequencies were tested using 2×2 contingency tables for each allele and combined the remaining alleles, and a *P*-value of less than $0.05/n$ was considered significant to correct for the number of comparisons made (Bonferroni correction).[19]

Results

We successfully identified the genotypes of more than 92% of the total 18 144 genotypes found in 63 microsatellites from the 288 study subjects. The observed and expected frequencies of each genotype for all 63 microsatellites in the NT group were in Hardy–Weinberg equilibrium (data not shown). We identified three loci showing significant ($P \leqslant 0.05$) among the 63 loci (Table 1). The *P*-values of D1S507, D1S2713 and D1S2842 were 0.037, 0.035 and 0.018, respectively.

Table 1 Association study of microsatellites

Microsatellite	χ^2 *value*	P-*value*
D1S468	11.2	0.189
D1S2660	5.8	0.218
D1S214	6.0	0.640
D1S450	12.9	0.115
D1S2667	7.7	0.263
D1S434	3.7	0.595
D1S507	17.8	0.037*
D1S2697	1.0	0.915
D1S2644	4.8	0.573
D1S199	8.0	0.331
D1S2864	7.6	0.177
D1S234	13.8	0.131
D1S233	16.5	0.057
D1S255	4.5	0.215
D1S2892	5.9	0.554
D1S2713	13.6	0.035*
D1S2797	11.6	0.071
D1S2652	5.7	0.332
D1S2890	10.5	0.398
D1S2873	1.0	0.963
D1S2737	5.6	0.588
D1S2846	6.7	0.565
D1S230	12.6	0.081
D1S198	5.2	0.637
D1S2841	3.4	0.842
D1S500	2.7	0.753
D1S207	6.3	0.609
D1S2766	10.8	0.096
D1S435	2.0	0.851
D1S2868	2.7	0.753
D1S2793	7.0	0.221
D1S206	4.7	0.700
D1S495	7.4	0.691
D1S2726	2.8	0.584
D1S252	9.9	0.194
D1S498	6.2	0.289
D1S2635	8.1	0.233
D1S484	9.4	0.051
D1S2878	11.9	0.156
DIS196	5.1	0.167
D1S452	1.6	0.815
D1S218	6.4	0.606
D1S2818	4.0	0.412
D1S238	5.5	0.601
D1S2877	1.4	0.711
D1S412	6.5	0.371
D1S413	12.3	0.056
D1S249	14.0	0.175
D1S2692	8.7	0.366
D1S245	4.5	0.722
D1S425	11.5	0.073
D1S227	1.4	0.711
D1S213	6.4	0.607
D1S2833	3.4	0.909
D1S2709	1.4	0.494
D1S2800	5.6	0.585
D1S2850	6.1	0.190
D1S2670	7.3	0.504
D1S2785	11.1	0.086
D1S304	3.5	0.174
D1S2842	15.3	0.018*
D1S423	0.7	0.719
D1S2836	9.5	0.391

The list of the microsatellites reflects the location on chromosome 1. D1S468 is located on the telomere side of 1p, D1S2836 is on the telomere side of 1q.
*$P<0.05$.

Allelic distributions of the three loci with the *post hoc* cell contributions for each cell are shown in Table 2. The *P*-values of the allele showing the highest *post hoc* cell contributions for D1S507, D1S2713 and D1S2842 were 0.0008, 0.0062 and 0.0084, respectively. All these values were significant after Bonferroni correction. In the tree microsatellite markers, five alleles showing significant differences of frequencies between the two groups were discriminated (Table 2). By comparing the clinical data in each allele, it was found out that allele D of D1S507, allele I of D1S2713 and allele J of D1S2713 were associated with the levels of systolic blood pressure (Table 3). The levels of systolic and diastolic blood pressures showed a significant difference after adjusting for age between the groups with allele I of D1S2713 and without the allele.

Discussion

Genetic markers that are sufficiently polymorphic (as measured by their heterozygosities) can be used in linkage and association analyses to detect Mendelian segregation underlying disease phenotypes. Each type of analysis can either be based on a specific genetic model or not make any assumptions about the mode of inheritance of the disease. The principles underlying these methods are reviewed, and the assumptions underlying them stressed. There is still much debate as to the methods of genetic dissection for complex diseases. The major success of affected sib-pair method for angiotensinogen gene polymorphism associated with EH[12] notwithstanding, linkage analysis has limited power to detect genes of modest effect.[21,22] A case–control study has far greater power and may be more appropriate for detecting genes involved in complex diseases.[23] Furthermore, a case–control study has an advantage to collect samples because EH develops in the later stage of life. In general, following the establishment of an association, family-based linkage-disequilibrium tests, such as the transmission-disequilibrium test (TDT),[24] offer an independent means to assess the association, which control for the confounding effects of population stratification or admixtures that plague population-based association tests.

The availability of microsatellite markers distributed throughout the genome together with the development of PCR-based semiautomated scoring techniques have made linkage studies for human genetic disease a practical approach. While microsatellite procedures are labour intensive, technological advances promise to make SNP screens highly automated and faster. As each SNP site is biallelic, and therefore less informative than a variable microsatellite that has multiple alleles, more SNP loci are needed to be equally informative. The high frequency of SNPs in the genome provides enough polymorphic sites to more than compensate for the

Table 2 Allelic distributions of the three loci identified in the association study

Locus	*D1S507*				*D1S2713*				D1S2842			
Alleles	*EH No.*	*NT No.*	Post hoc *cell contributions*	P-*value in 2 × 2 contingency table*	*EH No.*	*NT No.*	Post hoc *cell contributions*	P-*value in 2 × 2 contingency table*	*EH No.*	*NT No.*	post hoc *cell contributions*	P-*value in 2 × 2 contingency table*
A	2	3	—	NS	0	1	—	NS	1	0	—	NS
B	5	2	1.1	NS	2	2	—	NS	1	7	2.1	NS
C	35	38	0.6	NS	5	7	0.8	NS	121	109	1.2	NS
D	74	40	3.4[a]	0.001	80	63	0.9	NS	18	9	1.8	NS
E	11	14	0.7	NS	117	113	0.8	NS	16	10	1.2	NS
F	31	27	0.4	NS	15	17	0.7	NS	64	63	0.2	NS
G	4	1	—	NS	3	1	—	NS	46	72	26[a]	0.049
H	19	24	0.9	NS	9	5	0.9	NS	5	4	0.4	NS
I	67	89	2.4	0.015	25	8	2.7[a]	0.007				
J	15	10	0.9	NS	14	24	2.1	0.039				
K	6	10	1.1	NS	2	1	—	NS				
L	4	3	0.3	NS								
M	1	1	—	NS								

EH; essential hypertension, NT; normotension.
[a]The allele showing the highest *post hoc* cell contributions. NS; not significant.

Table 3 Clinical data in each genotype

	D1S507			*D1S507*			*D1S2713*			*D1S2713*			*D1S2482*		
	Allele D+	*Allele D–*	P *value*	*Allele I+*	*Allele I–*	P *value*	*Allele I+*	*Allele I–*	P *value*	*Allele J+*	*Allele J–*	P *value*	*Allele G+*	*Allele G–*	P *value*
Number of subjects.	100	175		132	143		29	228		34	223		92	177	
Age (years)	53.5±9.3	51.8±9.7	0.166	52.1±9.5	52.7±9.7	0.644	57.7±10.9	52.1±9.2	0.003	49.9±7.3	53.2±9.8	0.061	53.4±9.8	52.2±9.5	0.307
BMI (kg/m^2)	23.8±3.2	23.2±3.2	0.127	23.2±3.6	23.6±2.8	0.339	23.1±3.3	23.3±3.2	0.743	23.5±3.9	23.3±3.1	0.758	23.1±3.4	23.4±3.1	0.582
SBP (mmHg)	150.0±32.2	137.3±33.8	0.003*	138.8±30.9	144.8±36.0	0.142	160.4±345	141.5±33.1	0.004*	129.6±30.3	145.8±33.8	0.009*	137.0±31.5	144.9±34.4	0.065
DBP (mmHg)	90.9±20.7	83.0±19.0	0.002*	84.8±19.2	86.9±20.6	0.373	94.0±16.0	86.0±20.5	0.043*	81.8±20.0	87.7±20.1	0.112	83.1±19.4	87.6±20.2	0.080
Pulse (beats/min)	75.1±10.6	74.3±14.5	0.630	74.6±14.3	74.6±11.9	0.994	73.5±11.0	75.0±13.8	0.605	71.9±12.6	75.3±13.6	0.241	73.3±12.1	74.9±12.9	0.384
Creatinine (mg/dl)	0.8±0.2	0.8±0.3	0.306	0.9±1.0	0.8±0.2	0.406	0.8±0.2	0.8±0.3	0.592	0.8±0.2	0.8±0.3	0.874	0.8±0.2	0.8±0.3	0.902
Total cholesterol (mg/dl)	210.3±32.8	203.3±42.5	0.163	201.9±39.9	209.4±38.6	0.124	208.1±44.1	206.7±39.0	0.863	199.3±32.2	208.1±40.5	0.228	209.3±35.0	204.9±41.0	0.394
HDL cholesterol (mg/dl)	59.8±30.6	58.1±16.8	0.580	57.4±17.7	60.0±26.8	0.368	57.2±19.8	58.7±23.9	0.760	52.1±14.0	59.5±24.5	0.108	58.2±19.2	59.1±24.5	0.789
Uric acid (mg/dl)	5.5±1.6	5.4±1.4	0.683	5.3±1.5	5.6±1.5	0.967	5.4±1.3	5.5±1.5	0.868	5.2±1.2	5.5±1.5	0.323	5.6±1.4	5.4±1.5	0.462
Alcohol consumption (%)	60.2	66.7	0.315	59.3	69.1	0.113	58.3	63.4	0.630	76.9	61	0.114	69.6	60.5	0.170
Smoking (%)	39.8	49.7	0.132	50	42.1	0.214	36	45.6	0.363	60.7	42.3	0.066	47.6	44.9	0.699

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; NT, normotension; EH, essential hypertension.
*P<0.05.

lost information content. Genome scans commonly test 300–400 polymorphic microsatellites, spaced at 10 cM (10% recombination) intervals. The minimum number of moderately variant sites to obtain equivalent linkage power is 700–900 and a preliminary genome screen with a marker density of one per cM would require in the order of 1500–3000 SNPs.[25]

An increasing number of reports of genome scans for hypertension and BP variation have been seen in the past few years.[3–7,26–28] Most of the scans report nominal or suggestive linkages, although a few studies report significant linkages.[29–32] It is notable that two of these four studies have been conducted in a relatively isolated populations,[31,32] where the genetic homogeneity is likely to be much higher. Genome scan studies in populations with high genetic homogeneity also help narrow down the genome regions containing the loci of interest; to date, the smallest region suspected of harbouring a locus affecting the risk of hypertension is a 0.54-cM region on chromosome 2 by Angfius *et al*[32] in an isolated Sardinian population. A number of regions, notably those on chromosomes 1q, 2p, 3p, 6q, 7q, 11q, 12q, 15q, 16q, 18q and 19q, have been found in more than one study, strengthening the likelihood that such regions harbour BP-modifying loci.[2] However, these regions are broad and it is possible that these overlaps are due to different genes or false-positive linkages.[33] Recently, the Medical Research Council British Genetics of Hypertension (BRIGHT) study reported that some candidate loci for EH were identified by the whole genomewide scan in 2010 affected sibling pairs drawn from 1599 severely hypertension families using microsatellite markers.[34] It showed that four loci on chromosome 6q, 2q, 5q and 9q attained genomewide significance. BRIGHT is sib-pair analysis using the Linkage Marker Set MD10 (Applied Biosystems) having markers distributed at an average marker density of 10 cM, while our study is a case–control study using markers with an average spacing of 4.5 cM.

Not surprisingly, when different studies are compared, different results are obtained. One explanation for this divergence is that different analytical methods, genetic maps and markers have been used. Another explanation for the divergence in results between studies is that EH is a multifactorial and heterogeneous disease, presumably with different contributing susceptibility factors. Striking differences in incidence, prevalence and the clinical patterns among different ethnic populations reported in several epidemiological studies support this view.

In the several reports of genome scans including projects of large-scale networks, chromosome 1q showed a suggestive linkage in the GenNet study, and also has parallels with other studies.[4,6] We used 63 microsatellite markers to analyse chromosome 1 in 144 Japanese EH patients and NT subjects. We identified three loci with significantly different allelic distribution between the EH and NT groups: D1S507 located on 1p36, and matched to loci IBD7 (Bone mineral density variability 3) and KIAA1026 (KIAA1026 protein); D1S2713 located on 1p34 with no matches to other loci (GeneBank search); and D1S2842 located on 1q42.3–q43, and matched to loci PCAP (predisposition for prostate cancer). Although these matching peptides are unlikely to be associated with controlling BP, it was interesting that D1S2842 showed the most significant *P*-value and was located on 1q42–q43, the same locus as the angiotensinogen gene. The M235 T variant angiotensinogen gene, in particular, has been found to be associated with hypertension in several, although not all, studies.[12]

To our knowledge, there have been no reports of association studies using microsatellites for genome scans of multifactorial diseases. Therefore, we set the average distance between each marker at 4 cM, the same level used in familial genomewide scans. Even though our study is limited to a scan of chromosome 1, this serves as a first trial of this method.

In conclusion, we identified at least three susceptibility loci of EH on chromosome 1. This is the first study to assess the method of case-controlled genomewide scans using microsatellite markers. This method holds great promise for isolating the susceptibility loci of multifactorial disorders, and will be widely useful in the genomic research in the near future.

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