Original Article

Genome-Wide Linkage Disequilibrium Mapping of Hypertension in Japan

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Hypertension is a common, complex phenotype resulting from the interaction between genetic and environmental factors. To select candidate regions potentially responsible for hypertension, we are conducting a genome-wide linkage disequilibrium (LD) mapping of hypertension using dinucleotide repeat markers in 146 hypertensive and 136 normotensive subjects. Although the LD mapping is still underway, 19 alleles of 15 markers have already shown a nominally significant association ($p < 0.05$), with odds ratios ranging from 0.08 to 5.12, suggesting the presence of many hypertension-related loci with weak effects in the human genome. These markers should be further assessed, adjusting for confounding factors and considering gene-gene and gene-environmental interactions in additional samples. In this report, we discuss our ongoing LD mapping project and describe the 15 markers thus far discovered. Among the 15 markers, D10S537 had a highly significant association with hypertension ($p = 5.3 \times 10^{-5}$; OR = 3.80; 95% CI = 1.98–7.27; where OR indicates the odds ratio and 95% CI indicates the 95% confidence interval). Further analysis in a large Japanese population showed that D10S537 was significantly associated with hypertension ($p = 0.044$; OR = 1.27; 95% CI = 1.01–1.59). D10S537 was more significantly associated with hypertension in subjects with normotriglyceridemia in our population ($p = 0.007$; OR = 1.47; 95% CI = 1.11–1.95). (*Hypertens Res* 2003; 26: 533–540)

Key Words: hypertension, linkage disequilibrium mapping, microsatellite

Introduction

Hypertension is a common, complex disorder that results from interactions between genetic and environmental factors and has been intensively studied to identify susceptibility loci. One widely used strategy for identifying candidate polymorphisms for hypertension is the candidate gene approach. And in fact, polymorphisms in many candidate genes have been tested for their association with hyperten-

sion. However, association studies have reached divergent conclusions (1), and even the conclusions of meta-analyses have been inconsistent (2–5). Moreover, the candidate gene approach in large Japanese populations has also failed to show conclusive results (6–17). Thus, there is currently no genotypic polymorphism that has been proven to be associated with hypertension in humans.

Another strategy to search for candidate polymorphisms for hypertension is linkage mapping in humans, and large-scale linkage mappings of hypertension have led to the iden-

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Table 1. Characteristics of Participants in Linkage Disequilibrium Mapping

	First group		Second group		Third group	
	HT1	NT1	HT2	NT2	HT3	NT3
Number of subjects	48	48	48	48	50	40
Age (years)	53.1 (6.3)	55.5 (2.5)	54.1 (5.1)	54.8 (3.0)	54.1 (4.0)	55.8 (7.5)
Number on treatment	17	0	17	0	25	0
SBP (mmHg)	162.5 (12.1)	111.1 (6.4)	156.4 (9.0)	114.3 (5.5)	160.4 (9.1)	111.3 (5.5)
DBP (mmHg)	85.7 (8.4)	77.7 (9.1)	81.0 (11.3)	73.8 (10.4)	80.7 (10.7)	73.0 (8.8)

Data are mean (SD). Each number following HT or NT means group number. HT, hypertensive subject; NT, normotensive subject; SBP, systolic blood pressure; DBP, diastolic blood pressure.

tification of several candidate regions (18–29). However, no region showed highly significant linkage according to a proposed criterion (30), and the candidate regions have been inconsistent, suggesting that the development of hypertension may not be attributable to a few strong genetic factors. Instead, many weak-to-moderate genetic factors may contribute to the development of hypertension, although the number of such genetic factors and their relative risks remain unknown. Indeed, the authors of a large-scale linkage study concluded that the lack of a highly significant maximum logarithm of odds score values in their study demonstrated just how difficult genome-wide searches for genes influencing complex traits can be (9). This difficulty may be attributable to several characteristics of linkage analysis. First, if we assume that many weak-to-moderate genetic factors may contribute to the development of hypertension, then the relatively weak statistical power of linkage analysis could fail to identify them. Second, the results of linkage analysis could be influenced by wide regions of chromosomes over the range of linkage disequilibrium (LD), and such wide regions may contain more than several genetic factors influencing the development of hypertension. For example, at least three functional polymorphisms can exist in relation to hypertension even within a single gene, as has been reported for the β -2 adrenergic receptor gene (31, 32). The interpretation of the results may therefore be complicated, and the statistical power of linkage analysis to find a single genetic factor may be even weaker. Finally, linkage analysis has proven difficult to conduct when considering a number of gene–environmental and gene–gene interactions, despite the fact that the development of hypertension is considered to be at least partly attributable such interactions (33).

Owing to the complex nature and late onset of common disorders, LD mapping of genes in population samples has been suggested as an alternative to conventional linkage mapping. Indeed, large-scale LD mappings of Alzheimer's disease have identified several candidate loci (34, 35). However, to date, no large-scale LD mapping of hypertension has been conducted. We therefore planned to conduct an efficient large-scale LD mapping of hypertension using dinucleotide repeat markers. And although this mapping is still underway, 15 markers have already been found to be nominally associ-

ated with hypertension. In this report, we discuss our ongoing LD mapping project and describe the 15 markers thus far discovered.

Methods

Subjects

According to the criteria described below, 146 hypertensive and 136 normotensive subjects were selected from a population composed of 2,426 subjects who worked in a company in the Ehime region of Japan. All subjects were Japanese. They participated in medical check-ups 1 to 11 times (average, 6.2 times per person), and the mean values of variables in their personal health records were used in analyses. These 146 hypertensive and 136 normotensive subjects were further assigned to one of three groups (Table 1) according to the available amount of DNA. For each of the six subgroups thus created, a DNA pool was established by taking an equal amount of genomic DNA from each member. All subjects gave their informed consent and the study was approved by the ethics committee of Ehime University.

Diagnostic Categories

In our view, a case-control study should be performed in order to sensitively screen candidate polymorphisms for hypertension. In contrast, a population study should be performed in order to assess the extent to which candidate polymorphisms affect the development of hypertension, with the goal of establishing a genetic diagnosis. Therefore, in the genome-wide LD mapping of hypertension, we set very strict criteria for hypertensive subjects but relaxed criteria for statistical significance, whereas in our population study, we set relaxed criteria for hypertensive subjects with stringent criteria for significance. The criteria for hypertensive subjects in the genome-wide LD mapping of hypertension were defined as follows (Table 1): 1) male; 2) age < 60 years; and 3) systolic blood pressure (SBP) $\geq$ 160 mmHg without hypertensive medication or SBP $\geq$ 150 mmHg with antihypertensive medication. The criteria for normotensive subjects in the genome-wide LD mapping of hypertension

Table 2. Characteristics of Participants in Population Study According to Hypertensive Status

Variable	Normotensive (n = 1,687)	Hypertensive (n = 739)
Sex (male %)	86.4	89.3
Age (years)	51.6 (7.7)	55.2 (6.1)
Body mass index (kg/m ²)	22.8 (2.7)	24.4 (3.0)
SBP (mmHg)	123.8 (9.7)	150.9 (9.9)
DBP (mmHg)	72.1 (6.3)	87.7 (6.3)
Total cholesterol (mg/dl)	196.7 (31.6)	202.9 (31.6)
HDL cholesterol (mg/dl)	60.4 (13.3)	60.4 (13.6)
Triglyceride (mg/dl)	130.9 (75.8)	156.2 (85.1)
Smoking (heavy smoker %)	28.5	24.6
Alcohol (moderate to heavy drinker %)	29.2	38.2

HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure. Data are mean (SD). Blood pressure readings prior to the start of antihypertensive treatment were not available for 141 hypertensive subjects whose values were measured under treatment.

were defined as follows: 1) male; 2) age between 49 and 60 years; 3) SBP $\leq$ 115 mmHg; and 4) no history of angina pectoris or myocardial infarction. In the population study, the criteria for hypertensive and normotensive subjects were defined as follows (Table 2). Hypertensive subjects had previously been diagnosed with hypertension and were being treated with antihypertensive medication, or their SBP/diastolic blood pressure (DBP) was $\geq$ 140/90 mmHg. Normotensive subjects had never been treated with medication for hypertension, and their SBP/DBP was $<$ 140/90 mmHg. Hypertriglyceridemia was defined as triglyceride $\geq$ 150 mg/dl (Japan Atherosclerosis Society, Tokyo, Japan).

DNA Analysis

DNA was extracted from whole blood with a QIAamp Blood Kit (Qiagen K.K., Tokyo, Japan). Multiplex fluorescent-based genotyping was performed using the ABI Prism Linkage Mapping Set, HD-5 (PE Biosystems, Foster City, USA). Polymerase chain reaction (PCR) analysis was used to genotype dinucleotide repeat markers. Sizes of the PCR products were determined with an ABI 3100 genetic analyzer (PE Biosystems). Peak heights derived from electropherograms of pooled DNA amplifications were converted to 96 allele-frequency counts. The average spacing between markers was 5 centimorgans (cM).

Statistical Methods

All statistical analyses were performed on a personal computer using SPSS software (Version 10.0J for Windows; SPSS Inc., Chicago, USA). Categorical variables were compared using the χ^2 statistic or Fisher's exact test, as appropriate.

Logistic regression models were used to assess whether the polymorphism made a statistically significant contribution to prediction of hypertension, with consideration of interactions between the polymorphism and confounding factors. Sex, age, body mass index, plasma total cholesterol, high density lipoprotein-cholesterol, triglyceride levels, smoking status, and alcohol consumption were considered to be confounding factors (Table 2). Logarithmically transformed plasma triglyceride values were used in the analyses. General linear regression models were used to assess whether the polymorphism made a statistically significant contribution to prediction of blood pressure, with consideration of interactions between the polymorphism and confounding factors. A probability (*p*) value of less than 0.05 was considered statistically significant.

Results

Genome-Wide Linkage Disequilibrium Mapping of Hypertension

To conduct a large-scale LD mapping of hypertension efficiently, we used a multi-layered design and pooled DNA screening. First, 453 of 811 dinucleotide repeat markers were genotyped using the pooled DNA of the first subgroup and analyzed for an allelic association with hypertension, whereas the other 358 markers remain to be genotyped. As a result, 205 of 453 markers showed a nominal association. Subsequently, 203 of the 205 markers were genotyped and analyzed using the pooled DNA of the second group, with the other two markers remaining to be genotyped. As a result, 151 of 203 markers showed a nominal allelic association in the group made up of the first and second groups combined. After this rough screening of markers associated with hypertension using pooled DNA, 77 of the 151 markers were individually genotyped and analyzed in the group made up of the first and second groups combined. The other 74 markers remain to be genotyped. As a result, 28 of the 77 markers were confirmed to show a nominal allelic association in the group made up of the first and second groups combined. Further, 23 of the 28 markers were genotyped in the third group and analyzed in the group made up of the first, second, and third groups, whereas the other five markers remain to be genotyped. As a result, 15 of the 23 markers showed a nominal allelic association in the group made up of the first, second, and third groups (Table 3). Thus, although the LD screening of hypertension is still underway, 15 markers showed a nominal allelic association in the group made up of the first, second, and third groups combined, as well as in the group made up of the first and second groups combined.

Subsequent comparisons of individual allele frequencies at these loci between the hypertensive and normotensive subjects revealed only one allele at each locus showing significance at 11 loci and two alleles at each locus showing

Table 3. Summary of Alleles Associated with Hypertension in Combined Group of First, Second, and Third Groups

Maker	Locus	No. of alleles	Allele*	Allele frequency		OR	95% CI	<i>p</i> value
				HT	NT			
D1S2667	1p36.23	10	3	0.369	0.259	1.67	1.16–2.40	0.006
D1S2667	1p36.23	10	8	0.075	0.143	0.48	0.28–0.84	0.010
D1S2785	1q43	10	8	0.195	0.117	1.82	1.14–2.92	0.013
D2S2163	2p16.3	10	3	0.415	0.309	1.59	1.11–2.28	0.011
D3S1581	3p21.31	14	11	0.004	0.045	0.08	0.02–0.38	0.002
D4S2912	4p15.1	11	5	0.040	0.008	5.12	1.29–20.33	0.020
D4S2912	4p15.1	11	8	0.135	0.224	0.54	0.34–0.86	0.009
D5S630	5p15.2	24	3	0.187	0.120	1.68	1.03–2.74	0.036
D7S493	7p15.3	19	14	0.026	0.066	0.37	0.16–0.88	0.024
D7S493	7p15.3	19	15	0.004	0.031	0.11	0.64–0.02	0.014
D7S515	7q22.1	12	3	0.031	0.000	—	—	0.006
D7S515	7q22.1	12	4	0.069	0.132	0.49	0.27–0.89	0.019
D9S288	9p24.2	13	3	0.049	0.014	3.57	1.07–11.88	0.038
D10S537	10q22.2	10	8	0.138	0.040	3.80	1.98–7.27	5.3×10^{-5}
D10S217	10q26.3	12	7	0.100	0.190	0.48	0.29–0.79	0.004
D13S1320	13q21.1	8	3	0.019	0.068	0.27	0.10–0.71	0.008
D14S1051	14q32.33	7	1	0.939	0.888	1.94	1.04–3.63	0.037
D16S520	16q24.3	10	4	0.179	0.103	1.90	1.12–3.25	0.018
D16S3075	16p13.3	9	3	0.062	0.121	0.48	0.26–0.88	0.018

* Marker allele showing association in single-allele test. HT, hypertensive subject; NT, normotensive subject; OR, odds ratio; CI, confidence interval.

Table 4. D10S537 Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype and allele	Genotype frequency		<i>p</i> value	OR	95% CI
	Normotensive (<i>n</i> = 1,687)	Hypertensive (<i>n</i> = 739)			
D10S537 genotypes					
Allele 8 carrier	251 (14.9%)	134 (18.1%)	0.044	1.27	1.01–1.59
Allele 8 non-carrier	1,436 (85.1%)	605 (81.9%)			
D10S537 alleles					
Allele 8	260 (7.7%)	138 (9.3%)	0.057	1.23	0.99–1.52
Other alleles	3,114 (92.3%)	1,340 (90.7%)			

OR, odds ratio; CI, confidence interval.

significance at the other four loci, resulting in detection of a total of 19 alleles showing significance (Table 3). This suggests the presence of 19 hypertension-related loci in close proximity to the 15 loci. The levels of statistical significance of the 19 alleles ranged from 0.038 to $10^{-5} \times 5.3$. Of the 19 alleles, 10 alleles appeared to have a risk-increasing effect on the development of hypertension, while nine alleles appeared to have a protective effect against the development of hypertension. The odds ratios (ORs) of the 19 alleles ranged from 0.08 to 5.12. It should be noted that in this LD screening of hypertension, we could detect an OR of 2.6 with 80% power at a 5% type I error probability.

Association of D10S537 with Hypertension in the General Population

D10S537 showed a highly significant association with hypertension among the 15 markers thus far selected through the ongoing genome-wide LD mapping (Table 3). We therefore analyzed the association between D10S537 and hypertension in the whole population from which the 146 hypertensive and 136 normotensive subjects were selected. This analysis showed that D10S537 was significantly associated with hypertension even in the whole population ($p = 0.044$; OR = 1.27; 95% CI = 1.01–1.59; where 95% CI indicates 95% confidence interval) (Table 4). Adjustment for all confounding factors showed a non-significant but similar trend ($p = 0.069$; OR = 1.26; 95% CI = 0.98–1.62). This associa-

Table 5. Association of D10S537 Genotype with Hypertension According to TG Status

Genotype	Genotype frequency		<i>p</i> value	OR	95% CI
	Normotensive (<i>n</i> = 1,687)	Hypertensive (<i>n</i> = 739)			
Normotriglyceridemia (<i>n</i>)					
Allele 8 carrier (272)	184 (14.9%)	88 (20.5%)	0.007	1.47	1.11–1.95
Allele 8 non-carrier (1,392)	1,051 (85.1%)	341 (79.5%)			
Hypertriglyceridemia (<i>n</i>)					
Allele 8 carrier (113)	67 (14.8%)	46 (14.8%)	0.995	0.99	0.70–1.50
Allele 8 non-carrier (649)	385 (85.2%)	264 (85.2%)			

TG, triglyceride; OR, odds ratio; CI, confidence interval.

Table 6. Association of D10S537 Genotype with Blood Pressure According to TG Status

Genotype	SBP (mmHg)	DBP (mmHg)
Normotriglyceridemia (<i>n</i>)		
Allele 8 carrier (272)	132.3 (16.1)	76.7 (9.7)
Allele 8 non-carrier (1,392)	129.4 (15.3)	75.2 (9.1)
<i>p</i> value	0.005	0.016
Hypertriglyceridemia (<i>n</i>)		
Allele 8 carrier (113)	136.6 (15.2)	79.8 (9.3)
Allele 8 non-carrier (649)	136.8 (15.8)	79.9 (9.4)
<i>p</i> value	0.941	0.896
Total (<i>n</i>)		
Allele 8 carrier (385)	133.6 (16.0)	77.6 (9.7)
Allele 8 non-carrier (2,041)	131.8 (15.8)	76.7 (9.5)
<i>p</i> value	0.038	0.093

TG, triglyceride. Data are mean (SD). SBP, systolic blood pressure; DBP, diastolic blood pressure.

tion was pronounced in subjects with normotriglyceridemia ($p = 0.007$; OR = 1.47; 95% CI = 1.11–1.95) (Table 5). The association in subjects with normotriglyceridemia was significant even after adjustment for all confounding factors ($p = 0.049$; OR = 1.36; 95% CI = 1.00–1.84). In contrast, D10S537 was not associated with hypertension in subjects with hypertriglyceridemia ($p = 0.995$; OR = 0.99; 95% CI = 0.70–1.50).

More quantitatively, we further analyzed the association between D10S537 and blood pressure (Table 6). This analysis showed a significant association between D10S537 and SBP ($p = 0.038$). Adjustment for all confounding factors showed a non-significant but similar trend ($p = 0.101$). The analysis also showed a non-significant trend supporting the presence of an association between D10S537 and DBP ($p = 0.093$). After adjustment for all confounding factors, the p value was 0.260. Analysis of the association between D10S537 and SBP according to stratified triglyceride levels showed a significant association between D10S537 and blood pressure ($p = 0.005$ for SBP and $p = 0.016$ for DBP).

Adjustment for all confounding factors showed a non-significant but similar trend ($p = 0.058$ for SBP and $p = 0.169$ for DBP). In contrast, D10S537 was not associated with blood pressure in subjects with hypertriglyceridemia ($p = 0.941$ for SBP and $p = 0.896$ for DBP).

Discussion

We are presently conducting a large-scale LD mapping of hypertension using dinucleotide repeats and a multi-layered design. Two similar large-scale LD mappings of Alzheimer's disease have been conducted using tri- and tetranucleotide repeat markers (34, 35), and most of the markers were shown to overlap with each other. However, most of the markers that showed a significant association were different between the two mappings. This may have been attributable to the insufficient sample sizes in these mappings, and/or to their relaxed criteria for significance. Nevertheless, one of the markers associated with Alzheimer's disease in the first large-scale LD mapping of Alzheimer's disease (34), D10S1423, was repeatedly shown to be associated with Alzheimer's disease in ethnically different independent populations (36). Moreover, the region including this marker was coincidentally shown to be associated with Alzheimer's disease in the second large-scale LD mapping of Alzheimer's disease (37), although the region has never been detected in linkage mappings. In addition, in the case of diabetes mellitus, the insulin gene has been detected in association studies, but not in linkage mappings (30, 37, 38). Thus, linkage mappings may not be appropriate to find candidate regions influencing the development of common disorders with weak effects. In contrast, large-scale LD mappings may be potentially useful to select such candidate regions. However, large-scale LD mappings have several drawbacks, including the need to analyze a number of polymorphic markers in numerous subjects to increase their reliability.

In addition, large-scale LD mappings of hypertension are influenced by LD existing throughout a population. In this context, although the size of LD blocks is dependent on the loci, and the maximum size of LD blocks in Japanese appears to be larger than 2.3 cM (39), the mean size of LD

blocks in Japanese remains to be estimated. However, given the finding that the mean size of LD blocks in northern Europeans is unlikely to be larger than 100 kb (40), our large-scale LD mapping using markers with an average spacing of 5 cM is far from able to detect all of the hypertension-related loci, and thus further mappings using more densely spaced markers are warranted.

Each of the two large-scale LD mappings of Alzheimer's disease employed approximately 50 cases and 50 controls in their initial mappings (34, 35). The numbers of loci detected in their mappings were 6 and 22, respectively (34, 35, 41, 42). In apparent agreement with these numbers, our mapping of hypertension detected 15 loci that were nominally associated with hypertension (Table 3). However, our mapping is still underway, and if our current rate of loci detection were to continue, the resulting number of hypertension-associated loci in the human genome would likely be too high. This may suggest that our mapping contained some false positive results. On the other hand, our high rate of loci detection might be partly attributable to the complex nature of hypertension. For example, the functions of many organs have an effect on blood pressure including the heart, vasculature, kidneys, adrenal gland, thyroid, sympathetic nerves, and brain. Moreover, many non-genetic factors, including age, body weight, stress, smoking, alcohol consumption, and diet, influence blood pressure. The effects of such non-genetic factors on blood pressure are also thought to be modified by genetic factors.

As a result of our ongoing large-scale LD mapping, D10S537 was selected for its highly significant association with hypertension in selected hypertensive and normotensive subjects (Table 3). However, our large-scale LD mapping is only a screening based on a relaxed criterion for significance to lessen false negatives, and the results of large-scale LD mapping may include false positives. For this reason, the results should be further examined in general populations. In this context, D10S537 was associated with hypertension also in our population (Table 4), supporting the efficacy of the screening to some extent. However, the association in the population was relatively weak, despite the fact that the 146 hypertensive and 136 normotensive subjects being used in the screening were included in the population. This stresses the need for studies in additional general populations, although the weak association in the population may have been due to the difference between the severe criteria for hypertensive subjects in the genome-wide LD mapping of hypertension and the relaxed criteria for hypertensive subjects in the population study. Moreover, because obvious candidate genes for hypertension have not yet been identified in the close proximity to D10S537, the biological plausibility of the association between D10S537 and hypertension, particularly in subjects with normotriglyceridemia (Table 5), remains to be investigated; such an association could simply represent a false positive error. Nevertheless, gene hunters might consider an examination of the surrounding markers.

However, it will be worth having rigorous evidence in hand before undertaking positional cloning to avoid the unpleasant prospect of chasing a phantom locus (30). We therefore plan to examine D10S537 in additional populations with sufficient information on environmental factors.

The association between D10S537 and hypertension in subjects with normotriglyceridemia will require further consideration. In general, hypertriglyceridemia was associated with hypertension, although the mechanism remains obscure. Therefore, if a gene in close proximity to D10S537 is on a pathway from hypertriglyceridemia to hypertension, a polymorphism in the gene could alter the effect of hypertriglyceridemia on hypertension. If this is the case, allele 8 carriers might activate the pathway constantly, leading to a relatively constant level of blood pressure. In contrast, allele 8 non-carriers might activate the pathway depending on triglyceride levels, leading to a changing level of blood pressure depending on triglyceride levels.

Our large-scale LD mapping has additional limitations. First, because the markers used in the mapping are sparsely spaced, we likely missed many hypertension-related loci. More densely spaced markers should therefore be used in future studies. Moreover, LD is not complete even within an LD block. Microsatellite markers in LD with a functional polymorphism responsible for hypertension should therefore be less sensitive than the functional polymorphism itself in detecting an association with hypertension. Second, the sample size may have been insufficient to detect hypertension-related loci with minor effects on blood pressure or to show statistically conclusive evidence using a more strict *p* value corrected by the number of hypotheses tested. The detected markers should therefore be further examined in additional samples. Adjustment for confounding factors and analysis in the light of gene-gene and gene-environmental interactions could also be helpful to assess these results.

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