Original Article

Identification of Hypertension-Susceptibility Genes and Pathways by a Systemic Multiple Candidate Gene Approach: The Millennium Genome Project for Hypertension

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A multiple candidate-gene approach was used to investigate not only candidate genes, but also candidate pathways involved in the regulation of blood pressure. We evaluated 307 single nucleotide polymorphisms (SNPs) in 307 genes and performed an association study between 758 cases and 726 controls. Genes were selected from among those encoding components of signal transduction pathways, including receptors, soluble carrier proteins, binding proteins, channels, enzymes, and G-proteins, that are potentially related to blood pressure regulation. In total, 38 SNPs were positively ($p<0.05$) associated with hypertension. Replication of the findings and possible polygenic interaction was evaluated in five G-protein–related positive genes (GNI2, GNA14, RGS2, RGS19, RGS20) in a large cohort population (total $n=9,700$, 3,305 hypertensives and 3,827 normotensive controls). In RGS20 and GNA14, dominant models for the minor allele were significantly associated with hypertension. Multiple dimension reduction (MDR) analysis revealed the presence of gene–gene interaction between GNA14 and RGS20. The MDR-proved combination of two genotypes showed a significant association with hypertension ($\chi^2=9.93$, $p=0.0016$) with an odds ratio of the high-risk genotype of 1.168 (95% confidence interval [CI] [1.061–1.287]). After correction for all possible confounding parameters, the MDR-proved high-risk genotype was still a risk for hypertension ($p=0.0052$). Furthermore, the high-risk genotype was associated with a significantly higher systolic blood pressure (133.08±19.46 *vs.* 132.25±19.19 mmHg, $p=0.04$) and diastolic blood pressure (79.65±11.49 *vs.* 79.01±11.32 mmHg, $p=0.019$) in the total population. In conclusion, a systemic multiple candidate gene approach can be used to identify

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not only hypertension-susceptibility genes but also hypertension-susceptibility pathways in which related genes may synergistically collaborate through gene–gene interactions to predispose to hypertension. (*Hypertens Res* 2008; 31: 203–212)

***Key Words*: hypertension, candidate gene, association study, gene–gene interaction, pathways**

Introduction

Hypertension is one of the most common complex genetic disorders affecting a large population, with a genetic heritability ranging from 15% to 35% (*1–5*). Like other multifactorial human traits, hypertension is caused by the interaction of multiple risk genes and environmental risk factors (*1–5*).

Vigorous efforts have been made to identify genes for hypertension. The candidate gene approach is a cornerstone for identifying the hypertension-susceptibility gene(s). Several candidate genes have been selected on the basis of known biochemical or physiologic components related to blood pressure regulation. However, the results of these studies have not been consistent, and the positive findings have rarely been replicated. Several underlying causes have been postulated to explain the failure of these studies to reproduce the data on candidate gene(s), including the heterogeneity of cases and controls, population stratification, and gene–gene and gene–environment interactions (*1–5*). In addition, it has been demonstrated that genetic interactions at multiple loci rather than a variant of a single gene underlie the genetic basis of hypertension (*1–5*).

Hypertension is polygenic (*6–8*), with alleles at many different loci being suggested to contribute to the ultimate disease trait, and specific combinations of causative alleles may differ between individuals (*6–9*). Although several candidate genes have been associated with the development of hypertension, the mechanisms and genetic network underlying this disease remain unknown (*3*). It has been persuasively argued that the gene being assessed increases susceptibility for the disease, but its effect is not sufficient to cause the disease (*3*).

Accordingly, the strategy for the candidate gene approach needs to be changed to detect polygenetic factors with weak effects. There has been a trend towards the simultaneous evaluation of several polymorphisms in separate genes (*4*, *10*, *11*). Combinations of polymorphisms at several of these loci could steadily increase the odds ratio for predicting hypertension or a hypertensive intermediate phenotype (*12–16*). However, the above-referenced studies were directed at genes for which there was a published association with hypertension. To search for multiple novel genes interacting with each other, it would be rational to find a specific pathway in which the genes are involved. Dominiczak *et al*. (*17*) referred to "pathwayomics," meaning a cardiovascular continuum leading from the investigation of interrogation of multiple single nucleotide polymorphisms (SNPs) in genes for a specific pathway. No study has ever evaluated susceptibility pathways in which multiple genes interact with each other to cause hypertension.

In the present study, we evaluated multiple genes to identify not only candidate genes but also candidate pathways in which multiple genes are synergistically involved in the regulation of blood pressure. The study was performed one of the Millennium Genome Project. In 2000, a series of national cooperative projects were begun under the auspices of the Prime Minister's Millennium Project (*18*). The projects focus on bold technological innovations in three areas: informatization, the aging society and the environment. The identification of genetic variations linked to the development of hypertension is one of the leading missions of the Millennium Project. Four other diseases—diabetes mellitus, cancer, asthma, and Alzheimer's disease—have also been chosen as targets for gene search.

In the present study, we evaluated 307 SNPs in 307 genes, and performed an association study between 758 cases and 726 controls. Genes were selected from those related to signal transduction pathways that included receptors, soluble carrier proteins, binding proteins, channels, enzymes, and G-proteins, and that were possibly related to blood pressure regulation. A replication study of positive G-protein–related SNPs including possible gene–gene interactions was further performed in a larger, independent general population.

Methods

Subjects

Case-Control Study

The present study was performed as part of the Millennium Genome Project for Hypertension under the auspices of the National Millennium Project in Japan. Four centers throughout Japan, located in Asahikawa, Tokyo, Osaka, and Hiroshima, participated in the case-control study. Cases and controls were recruited at each center, using the same criteria.

Hypertensive subjects ($n=758$) had a previous diagnosis of hypertension at between 30 and 59 years of age and were being treated with antihypertensive medication or had a systolic blood pressure (SBP) $\geq$160 mmHg and/or diastolic blood pressure (DBP) $\geq$90 mmHg. They had a family history of hypertension in their parents and/or siblings. They were not obese (body mass index [BMI] $<$25 kg/m^2). Normotensive controls ($n=726$) aged more than 45 years were recruited from the same regions. They had never been treated with antihypertensive medication and their SBP was $<$120 mmHg and DBP $<$80 mmHg. They had no family history of hypertension.

Table 1. Clinical Characteristics of Cases and Controls

	Hypertensive cases	Normotensive controls
Number of subjects	758	726
Asahikawa	192	192
Tokyo	159	153
Osaka	238	189
Hiroshima	169	192
Male (*n* (%))	564 (74.4)	550 (75.8)
Age (years old)	59.0±11.0*	62.8±9.4
Body mass index (kg/m^2)	23.6±3.0*	22.7±2.9
Systolic blood pressure (mmHg)	163.5±24.6	115.9±12.0
Diastolic blood pressure (mmHg)	100.3±15.7	72.0±7.6
Antihypertensive medication (*n* (%))	499 (65.8)	

*$p<0.05$ *vs*. normotensive controls.

Table 1 summarizes the background characteristics of the cases and controls.

Replication Study

Replication of positive findings including gene–gene interactions was evaluated in a large, independent general population recruited from five cohorts throughout Japan (Ohasama, Yokohama, Shigaraki, Ehime 1 and Ehime 2) (*19–22*). Subjects aged ≥30 years were enrolled in the analysis. Measurement of blood pressure, an anthropometric and biochemical parameters was performed during a medical checkup. Table 2 summarizes the clinical characteristics of the population. In this population sample, hypertension was defined as an SBP ≥160 mmHg and/or DBP ≥90 mmHg, or current use of antihypertensive medication. Normotension was defined as SBP <140 mmHg and DBP <90 mmHg and no current use of antihypertensive medication. Among the total 9,700 subjects, 3,305 were defined as hypertensives and 3,827 were classified as normotensive controls.

All of the participants in the study were native Japanese. All participants received a full explanation of the purpose of the study, and written informed consent for the procedures was obtained. The entire protocol followed the guidelines for genomic research of the Japanese government and was approved by the ethical committees of all institutes participating in the project.

SNP Selection

Three hundred and seven SNPs of the following types were selected. 1) SNPs in genes encoding the components of signal transduction systems, including enzymes, channels, receptors, solute carriers, G-proteins, and binding proteins, that are potentially related to blood pressure regulation. SNPs in other genes of particular interest, such as collagens, growth factors, adhesion molecules, and hormones, were also evaluated. 2) From among these 307 SNPs, we selected one SNP from one gene, preferably in the promoter region or exons with the highest minor allele frequency in the Japanese population, was selected based on information published in the JSNP website (*23*) (http://snp.ims.u-tokyo.ac.jp/index.html). 3) To find novel susceptible genes, unpublished SNPs were selectively studied. All SNPs examined in the present study are listed in the Online Data Supplement 1 (it is up-loaded to J-STAGE).

Genotyping

DNA was extracted from white blood cells by the standard method, at each institute. The whole genome was amplified by degenerated oligonucleotide primer (DOP)–polymerase chain reaction (PCR) in the Core Laboratory of the Department of Geriatric Medicine, Ehime University. DOP-PCR was performed according to a previous report, with slight modifications (*24*, *25*). In brief, 10 ng genomic DNA was used as a template in a 100-μL reaction mixture containing 5 U polymerase (TaKaRa LA Taq; Takara Biomedicals, Tokyo, Japan), 2.5 mmol/L $MgCl_2$, 400 μmol/L dNTP, and 4 μmol/L DOP primer (5′-CCGACTCGAGNNNNNNATGTGG-3′; Sigma Genosys Japan, Ishikari, Japan). The amplification conditions consisted of an initial incubation at 93°C for 1 min; followed by 8 cycles of 93°C for 1 min, 30°C for 1 min, and 72°C for 3 min; followed by 28 cycles of 93°C for 1 min, 60°C for 1 min, and 72°C for 3 min. The initial denaturation for 8 min at 96°C was omitted. The quality of amplified DNA obtained by DOP-PCR was checked in all samples.

Genotyping for the case-control study was performed in the Human SNP Typing Center of Tokyo University (Department of Human Genetics) using automated fluorescence correlation spectroscopy (*26*), and in the Core Lab of the Department of Geriatric Medicine, Ehime University, by the TaqMan PCR method.

Genotyping for the replication population study was performed at Ehime University using a TaqMan SNP genotyping assay system. The structures of primers and probes are listed in the Online Data Supplement 2 (it is up-loaded to J-STAGE).

Statistical Analysis

For comparison of the baseline characteristics between cases and controls, Student's unpaired *t*-test for continuous data and χ^2 test for categorical data were used. Differences in genotype and allele frequency between cases and controls were evaluated by χ^2 test. In the present study, both recessive and dominant models for the minor allele were considered.

Gene–gene interaction was evaluated by the multiple dimension reduction (MDR) method developed by Ritchie *et al*. (*27*). Genes were evaluated with a specific model, either dominant or recessive. This method included a combined cross-validation and permutation-testing procedure that mini-

Table 2. Clinical Characteristics of General Population

	Total sample	Hypertensives	Normotensives	*p*
Number of subjects	9,700	3,305	3,827	
Ohasama	1,663	523	542	
Yokohama	2,196	425	1,201	
Shigaraki	2,190	831	842	
Ehime 1	848	335	339	
Ehime 2	2,803	1,191	903	$\chi^2=253$, $p<0.0001$
Male (%)	52.2	53.5	50.0	
Age (years old)	56.8±12.6	62.8±10.6	50.5±11.6	<0.0001
Body mass index (kg/m^2)	23.1±3.2	24.0±3.2	22.2±2.9	<0.0001
Systolic blood pressure (mmHg)	132.5±19.3	148.6±17.3	115.3±9.6	—
Diastolic blood pressure (mmHg)	79.3±11.4	88.0±11.0	71.3±7.9	—
Antihypertensive medication (%)	19.6	60.0	—	
Total cholesterol (mmol/L)	5.21±0.90	5.29±0.88	5.13±0.90	<0.0001
HDL cholesterol (mmol/L)	1.53±0.40	1.52±0.41	1.57±0.40	<0.0001
Triglyceride (mmol/L)	1.37±0.94	1.52±1.06	1.22±0.84	<0.0001
Blood glucose (mmol/L)	5.8±1.6	6.1±1.8	5.5±1.3	<0.0001
Current smoker (%)	34.9	35.5	34.5	
History of stroke (%)	2.2	4.3	0	
History of heart disease (%)	5.8	9.3	0	

HDL, high-density lipoprotein.

mizes false-positive results by multiple examinations of the data. Cross-validation divides the data into a training set and a testing set. In 10-fold cross-validation, the data are divided into 10 equal parts, and the model is developed based on 9/10 of the data (training set) and then tested on the remaining 1/10 of the data (testing set). This is repeated for each possible training set, and the resulting 10 prediction errors are averaged (*27*).

The genotype combination obtained by the MDR method was then re-evaluated with an χ^2 test in the total population. Furthermore, differences in SBP and DBP as quantitative traits between MDR-defined genotypes were evaluated by ANOVA. Values are expressed as the means±SD. Values of $p<0.05$ were defined as statistically significant.

Results

Case-Control Study

Sixty-five percent of hypertensive cases were receiving antihypertensive medication. There were no region-specific differences in the parameters examined.

Systemic Multiple Candidate Gene Analysis

Of the 307 SNPs, 13 showed *p* values less than 0.05 in genotype frequency, 23 showed a significantly different allele frequency between cases and controls, and 32 showed significant associations with hypertension in either a dominant or recessive model for the minor allele. In total, 38 SNPs were found to be positively associated with hypertension (Table 3).

In the subcategorized analysis, the positive rate was high in genes related to G-proteins and genes of solute carriers (Table 4). The positive rate of SNPs in genes encoding the Gα-subunit or RGS (5/12), which could possibly interact with each other, was significantly higher than those in other classes of genes (5/12 *vs*. 33/295, $\chi^2=6.2$, $p=0.013$).

Replication in a Large Cohort Population

Replication of the findings of the case-control study and possible polygenic interaction in the susceptible pathway was evaluated in G-protein–related genes in a large cohort population.

Table 2 summarizes the clinical characteristics of the cohort population sample, from which 3,305 hypertensive subjects and 3,827 normotensive controls were recruited. Allele and genotype frequencies of the five G-protein–related SNPs in the cohort population are summarized in Table 5. The genotypes of GNA14 showed significant associations with hypertension. In RGS20 and GNA14, dominant models for the minor allele were significantly associated with hypertension. Accordingly, the gene–gene interaction of G-protein–related genes was evaluated in GNA14 and RGS20.

Gene–Gene Interaction

MDR analysis showed that the combination of two SNPs, GNA14 and RGS20, had a testing accuracy of 0.526 and

Table 3. Genotype and Allele Frequencies and Odds Ratio of Dominant and Recessive Models of Minor Allele in 38 Positive SNPs

Gene symbol	A (minor)/ B	A frequency	Case (*n*)				Control (*n*)				Allele (A *vs*. B)		A dominant		A recessive		Genotype *p*
			AA	AB	BB	H-W *p*	AA	AB	BB	H-W *p*	Odds ratio	*p*	Odds ratio	*p*	Odds ratio	*p*	
ATP2B1	C/A	0.4654	151	379	216	0.515	177	341	186	0.409	0.862	0.045	0.88	0.281	0.76	0.026	0.078
ATP10C	A/G	0.3574	115	342	291	0.382	80	333	295	0.335	1.159	0.056	1.122	0.283	1.426	0.023	0.069
ATP2A3	G/A	0.3359	81	324	345	0.705	52	301	366	0.354	1.223	0.013	1.217	0.06	1.553	0.017	0.028
ATP10D	C/T	0.4296	132	347	271	0.253	161	326	232	0.024	0.838	0.018	0.842	0.119	0.74	0.022	0.052
PRKWNK1	G/A	0.4918	185	342	214	0.040	182	356	158	0.523	0.863	0.049	0.723	0.008	0.934	0.607	0.026
DLGAP2	A/C	0.4907	150	385	219	0.412	192	346	182	0.299	0.81	0.004	0.826	0.104	0.683	0.002	0.007
GUCA1C	T/C	0.33	71	318	338	0.762	102	322	294	0.362	0.8	0.005	0.798	0.034	0.654	0.009	0.014
CYP17	T/C	0.4678	149	340	251	0.085	156	364	187	0.399	0.827	0.011	0.7	0.002	0.891	0.368	0.008
PTPRT	C/T	0.4925	144	362	249	0.543	165	348	205	0.459	0.845	0.023	0.812	0.066	0.79	0.066	0.08
PPP1R1B	G/A	0.4479	144	362	231	0.919	172	334	182	0.449	0.812	0.006	0.728	0.013	0.729	0.013	0.021
KCNN1	A/G	0.339	73	303	367	0.369	77	316	302	0.677	0.848	0.039	0.787	0.024	0.875	0.437	0.078
HCN4	G/T	0.4795	158	342	244	0.063	160	356	195	0.918	0.875	0.073	0.774	0.026	0.929	0.559	0.081
KCNIP2	A/G	0.4571	162	332	238	0.025	156	355	186	0.588	0.885	0.103	0.756	0.016	0.986	0.909	0.041
KCNMB4	T/G	0.41	136	370	242	0.793	133	300	263	0.005	1.096	0.226	1.27	0.0305	0.941	0.65	0.042
CACNA2D2	G/A	0.4845	185	401	166	0.065	158	356	206	0.857	1.202	0.013	1.415	0.004	1.161	0.228	0.015
CACNA1E	T/A	0.4693	160	331	245	0.016	179	318	201	0.020	0.845	0.024	0.811	0.066	0.805	0.082	0.097
ACCN1	G/A	0.4877	204	367	180	0.553	162	344	208	0.387	1.213	0.009	1.304	0.025	1.271	0.048	0.036
PTHR1	A/G	0.433	143	364	209	0.495	134	302	233	0.047	1.12	0.139	1.296	0.025	0.996	0.979	0.056
CALCR	A/G	0.4483	170	350	222	0.157	131	350	229	0.893	1.147	0.066	1.115	0.337	1.314	0.036	0.108
ADORA1	T/C	0.4558	188	361	186	0.632	152	337	205	0.542	1.172	0.035	1.237	0.073	1.226	0.103	0.111
SLC13A1	G/A	0.4043	144	337	255	0.088	104	347	239	0.229	1.097	0.224	1	0.997	1.371	0.025	0.06
SLC2A11	T/C	0.4037	125	342	254	0.592	97	304	266	0.503	1.169	0.045	1.22	0.074	1.232	0.156	0.139
SLC21A6	T/C	0.3851	129	289	283	0.000	93	285	271	0.200	1.123	0.144	1.059	0.605	1.348	0.044	0.127
SLC26A8	C/T	0.2602	59	276	412	0.184	63	295	347	0.979	0.841	0.037	0.788	0.024	0.874	0.476	0.077
SLC22A7	A/G	0.3443	101	339	296	0.802	75	299	325	0.615	1.228	0.009	1.292	0.017	1.323	0.084	0.034
RGS19IP1	G/A	0.3804	102	306	291	0.143	122	299	242	0.082	0.828	0.017	0.806	0.053	0.758	0.058	0.066
RGS2	G/A	0.438	140	341	255	0.173	135	365	208	0.262	0.898	0.15	0.785	0.032	0.997	0.982	0.077
RGS20	C/G	0.3973	93	346	298	0.631	115	316	268	0.183	0.881	0.101	0.916	0.417	0.733	0.039	0.118
GNAI2	A/G	0.454	177	375	187	0.682	132	350	228	0.909	1.278	0.001	1.396	0.004	1.379	0.013	0.004
GNA14	T/C	0.4042	105	351	283	0.819	122	347	241	0.879	0.858	0.044	0.828	0.085	0.798	0.12	0.13
RAC2	T/C	0.435	157	340	207	0.439	126	304	228	0.172	1.186	0.028	1.273	0.038	1.212	0.152	0.087
FGF2	C/T	0.4713	143	361	240	0.727	163	312	209	0.029	0.88	0.09	0.924	0.489	0.761	0.034	0.105
COL4A1	G/A	0.4633	165	372	205	0.878	143	309	226	0.052	1.148	0.067	1.31	0.02	1.07	0.6	0.062
HLA-DMB	A/C	0.4947	159	362	217	0.727	189	331	176	0.201	0.823	0.009	0.813	0.081	0.737	0.013	0.03
CAST	A/G	0.4695	155	368	210	0.791	174	308	198	0.015	0.923	0.29	1.023	0.846	0.78	0.048	0.091
EXOSC3	G/A	0.485	150	362	218	0.99	178	322	187	0.033	0.852	0.013	0.878	0.2712	0.74	0.0168	0.055
ERCC1	A/C	0.4443	145	362	231	0.882	170	322	204	0.056	0.873	0.069	0.91	0.413	0.757	0.029	0.092
CHGA	T/C	0.3482	105	371	268	0.194	94	323	291	0.770	1.135	0.1	1.24	0.047	1.073	0.643	0.1366

H-W, Hardy-Weinberg equilibrium.

cross-validation consistency of 10/10 ($p=0.025$), by sign test (Table 6). An empirical permutation test with 1,000 replications showed that the combination was statistically significant ($p=0.01$). Therefore, we concluded the presence of a gene–gene interaction between GNA14 and RGS20.

The MDR-proved combination of GNA14 and RGS20 was further verified in the cohort population (Table 7). The combination of the genotypes of GNA14 and RGS20 showed significant association with hypertension in the population ($\chi^2=9.93$, $p=0.0016$). The odds ratio of the risk genotype (GNA14: CC+TC and RGS20: CC+CG) to the non-risk genotypes defined by the MDR method was 1.168 (95% confidence interval [CI] [1.061–1.287], $p=0.0016$). Since the prevalence of hypertensives and normotensives was signifi-

Table 4. Positive Rates

Classification	Positive/total	Positive rate
Enzymes	10/71	14.1
ATPase	4/12	33.3
Kinase	2/12	16.7
Hcy	0/3	0
Metalloproteinase	0/3	0
Guanylate cyclase	1/3	33.3
NO synthase	0/3	0
Channels	7/55	12.7
K channel	4/27	14.8
Ca channel	2/10	20.0
Cl channel	0/8	0
Receptors	3/36	8.3
Serotonin	0/4	0
Thyroid	0/3	0
Cholinergic	0/2	0
Solute carriers	5/31	16.1
Na related	1/8	12.5
Neurotransmitter	0/6	0
Glucose	1/3	33.3
G-protein	6/27	22.2
α-Subunit	2/7	28.6
RGS	3/5	60.0
rho	1/9	11.1
ras	0/4	0
Growth factors	1/13	7.7
VEGF	0/3	0
FGF	1/3	33.3
IGF	0/3	0
Cytokines	0/12	0
IL1	0/6	0
TNF	0/3	0
Binding proteins	0/9	0
Collagen	1/4	25.0
Coagulation	0/4	0
Adhesion molecules	0/3	0
HLA	1/2	50.0
Miscellaneous	4/39	10.3
Total	38/307	12.4

Hcy, homocysteine; RGS, regulator of G-protein signaling; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IL1, interleukin 1; TNF, tumor necrosis factor; HLA, human leukocyte antigen.

cantly different among regions, gene–gene interaction was further evaluated by a general linear model with the following parameters; sex, region, age, BMI, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and blood glucose. After correction for sex, region, and age, the MDR-proved high-risk genotype had a significantly higher risk for hypertension (odds ratio 1.16 [1.035–1.294], $p=0.01$). After correction for all these confounding parameters, the MDR-proved risk genotype was still a risk for hypertension (odds ratio 1.19 [1.053–1.340], $p=0.0052$).

The blood pressure levels of the combined genotype were further evaluated in the total population of the cohort ($n=9,700$) (Table 8). The risk genotype was associated with a significantly higher SBP and DBP. Even after correction for other confounding parameters, including region, sex, age, BMI, use of antihypertensive medication, total cholesterol, HDL cholesterol, triglycerides and fasting glucose, DBP in the high-risk genotype was still significantly higher compared with that in the non-risk genotype ($p=0.0294$).

Discussion

Hypertension is a polygenic disease in which individual genes contribute only a very weak genetic effect (*1–5*). Furthermore, epigenetic effects could play pivotal roles. Accordingly, a conventional candidate gene approach may limit the possibility of detecting the susceptible genes for hypertension, since the effect of a single gene might not always be detected in different populations. Several studies have evaluated multiple genes as candidate hypertension genes and evaluated gene–gene interactions among candidate genes (*10–16*). However, many of these genes evaluated so far were already reported as positive genes in a previous candidate gene study. The strategy we took in the present study was to search for not only susceptibility genes, but also susceptibility pathways for hypertension using a candidate gene approach. The results raised the possibility that many susceptibility genes play an indirect role and exist in numerous pathways involved in blood pressure regulation.

The candidate genes evaluated thus far include genes within the renin-angiotensin system, α-adrenergic and β-adrenergic receptors, and growth factors, as well as genes encoding enzymes and peptides involved in endothelial function and vasoactivity (*17*). In the present study, we chose genes in signal transduction pathways possibly related to blood pressure regulation. The results revealed that none of the genes showed a high odds ratio or small p value.

Candidate SNPs were selected from among those in the gene of interest with the highest minor allele frequencies based on the JSNP data (*23*). The rationale behind this strategy is the common-disease/common-variant (CD/CV) hypothesis (*28*, *29*). Although several authors have argued that the CD/CV hypothesis has not been sufficiently validated (*5*, *30*, *31*), detection of susceptibility genes with low frequency is beyond the power of a conventional case-control approach, and at least this approach has the necessary statistical power to detect an association.

We demonstrated that positive SNPs were more common in genes related to G-proteins. We found that SNPs in genes that encoded ATPase, channels and solute carrier proteins also showed a higher positive rate. Among the positive pathways, replication of G-protein–related genes, including gene–gene interaction, was evaluated. Two of five positive G-protein–

Table 5. Five G-Protein Related SNPs in a General Population

Gene	Case (*n*)				Control (*n*)				Allele (A *vs*. B)		A dominant		A recessive		Genotype
	AA	AB	BB	H-W *p*	AA	AB	BB	H-W *p*	Odds ratio [95% CI]	*p*	Odds ratio [95% CI]	*p*	Odds ratio [95% CI]	*p*	*p*
RGS19	GG	GA	AA		GG	GA	AA								
	529	1,498	1,220	0.056	591	1,751	1,396	0.283	1.005 [0.94–1.08]	0.88	0.99 [0.90–1.09]	0.82	1.03 [0.91–1.17]	0.65	0.829
RGS2	GG	GA	AA		GG	GA	AA								
	603	1,520	1,104	0.049	663	1,837	1,224	0.563	0.99 [0.93–1.06]	0.78	0.94 [0.85–1.04]	0.24	1.06 [0.94–1.20]	0.34	0.179
RGS20	CC	CG	GG		CC	CG	GG								
	456	1,578	1,190	0.066	540	1,715	1,460	0.318	1.04 [0.97–1.12]	0.23	1.11 [1.004–1.22]	0.04	0.97 [0.85–1.11]	0.64	0.061
GNAI2	AA	AG	GG		AA	AG	GG								
	724	1,557	965	0.044	836	1,847	1,044	0.725	0.96 [0.90–1.03]	0.28	0.92 [0.83–1.02]	0.11	1.01 [0.88–1.14]	0.93	0.261
GNA14	TT	TC	CC		TT	TC	CC								
	542	1,611	1,111	0.302	629	1,757	1,386	0.072	1.06 [0.99–1.13]	0.11	1.13 [1.02–1.24]	0.018	0.99 [0.88–1.13]	0.94	0.040

H-W, Hardy-Weinberg equilibrium; CI, confidence interval.

Table 6. Multiple Dimensionality Reduction

	Gene(s)	Training accuracy	Testing accuracy	CV	Sign test consistency (*p*)	*p* value for testing accuracy by permutation test
One gene	GNA14	0.5044	0.491	6/10	3 (0.109)	0.93
Two genes	RGS20+GNA14	0.526	0.526	10/10	9 (0.0215)	0.014

Permutation test count 1,000, affected, ratio threshold was 0.868. CV, cross-validation.

related SNPs in the case-control study, GNA14 and RGS20, were also positively associated with hypertension in a replicated study in a large general population. Furthermore, possible gene–gene interaction was found between the two genes.

Heterotrimeric G-proteins are central components of the primary mechanism to receive, interpret and respond to a wide range of structurally and chemically diverse extracellular stimuli (*32*, *33*). Agonist binding of G-protein–coupled receptors (PCRs) promotes G-protein activation. This activation is achieved by catalyzing the GDP–GTP exchange on the α-subunit. A conformational change in the GTP-bound α-subunit leads to dissociation of Gα from the β- and γ-subunits. GTP-bound Gα-subunits and dissociated βγ-dimers regulate downstream effectors. These signaling events are terminated as a consequence of intrinsic GTPase activity of the Gα-subunit, which hydrolyzes bound GTP to GDP, resulting in reassociation of the G-protein heterotrimer (*34*). The intrinsic GTPase activity of α-subunits is generally insufficient to correlate with the physiological rate of G-protein inactivation, but this activity can be accelerated by the presence of GTPase-activating proteins (GAPs), such as regulator of G-protein–signaling (RGS) proteins (*35*).

Although the present study showed there is a statistically significant gene–gene interaction between GNA14 and RGS20, the details of this interaction remain to be clarified. Although GNA14 is much less promiscuous than GNA15 or GNA16, it has the ability to interact with several selective Gi-coupled receptors, such as α2-adrenergic, δ-opioid, ORL1 and SSTR3, to increase IP formation (*36*). On the other hand, it has also been shown that transfected RGSZ1 blocked mitogen-activated protein kinase activity induced by an α2-adrenergic receptor agonist (*37*). Taken together, these findings may indicate that GNA14 and RGS20 can interact through α2-adrenergic receptor. However, how the gene–gene interaction observed in the present study is functionally related to hypertension needs to be biologically determined.

In the present study, we used the MDR method to evaluate gene–gene interactions. The MDR method has been widely used for statistical mining of gene–gene and gene–environmental interactions (*38*, *39*). The dimensionality reduction approach seeks to identify combinations among multilocus genotypes that are associated with high risk of disease as well as combinations associated with low risk. Thus, MDR defines a single variable that incorporates information from several loci and/or environmental factors that can be divided into high-risk and low-risk combinations. This new variable can

Table 7. Multiple Dimensionality Reduction Method–Proved Risk Genotype and Hypertension in a General Population

	Risk genotype	Non-risk genotype
NT	1,401	2,277
HT	1,335	1,857

$\chi^2=9.93$, $p=0.0016$, odds ratio 1.168 [1.061–1.287]. Risk genotype: RGS20: CC+CG; GNA14: CC+TC. NT, normotensives; HT, hypertensives.

Table 8. MDR-Determined Genotype and Blood Pressure Values in General Population

	Non-risk genotypes	Risk genotype	*p*
n	5,625	3,667	
SBP (mmHg)	132.25±19.19	133.08±19.46	0.042
DBP (mmHg)	79.01±11.32	79.65±11.49	0.019

Model 2 (corrected for age and sex): *p* value of genotype for SBP is 0.131, and for DBP is 0.0295. Model 3 (further corrected for region, body mass index, use of antihypertensive drugs, total cholesterol, high-density lipoprotein cholesterol, triglyceride, and fasting glucose): *p* value of genotype for SBP is 0.187, and for DBP is 0.0294. MDR, multiple dimensionality reduction method; SBP, systolic blood pressure; DBP, diastolic blood pressure.

be evaluated for its ability to classify and predict disease risk status using cross-validation and permutation testing (*27*). The findings obtained by the MDR method need to be further confirmed not only in a separate population, but also by functional analysis.

The present study has several important limitations, including the threshold that was used to identify a positive gene (*40*, *41*). We used the statistical threshold of a *p* value less than 0.05. However, since we evaluated more than 300 genes at the same time, it would be have been more appropriate to use the probability threshold of Bonferroni correction—*i.e.*, $p<0.00016$ (=0.05/307) instead of 0.05. With this strict definition, there were no significant SNPs related to hypertension in the case-control study. However, since we used the present findings to further extend the research, it could be appropriate to adopt a weaker criterion to pick up positive SNPs. The statistics should be used as a guide to make decisions on whether further study would be necessary with the given probability (*40*).

To determine whether a finding with an observed *p*-value is noteworthy for publication, it has also been postulated that false-positive report probability should be evaluated based on the prior probability in the case-control association study with candidate genes (*42*, *43*). False-positive report probability has been shown to be influenced by statistical power, sample size, odds ratio, minor allele frequency, and *p* value of the association. In the present replication study, we had set moderate to low prior probability, *i.e.*, 0.1–0.01, since associations between genes and hypertension were already observed in the case-control study. The risk genotype obtained by the MDR method showed a false-positive report probability ranging from 0.02–0.2 with moderate to low prior probability, indicating that the observed positive findings are worthy of reporting (*43*).

The CD/CV hypothesis and common disease rare variant hypothesis have been debated (*28–41*). In the present study, candidate SNPs were selected on the basis of the common variant hypothesis. A multiple candidate gene approach for directing "pathwayomics" revealed that the effect of each SNP was small. Although exact replication of the original findings was not observed in the general population study, we did detect a gene–gene interaction which could have furthered increased the power of the effect of genes not only on hypertension but also on blood pressure variability. Again, however, the odds ratio was not high. Accordingly, the present findings were not consistent with the CD/CV hypothesis, since this hypothesis requires that the common variant should have at least a moderate effect on the phenotype (*28*). However, we could not eliminate the possibility that the common variants have only small effects and hypertension consists of numerous combinations of common variants with small effects.

In a study evaluating the effects of inbreeding on blood pressure as a quantitative trait within Croatian island isolates, a model of the distribution of locus effects suggested that the 8–16 quantitative trait loci (QTLs) of largest effect together account for a maximum of 25% of the dominance variation, while the remaining 75% of the variation is mediated by QTLs of very small effect, unlikely to be detectable using current technologies and sample sizes (*44*). The small but significant and independent effect on blood pressure variability observed in the high-risk genotype in the present study may support their findings. There is a possibility that numerous common genes with a small effect on blood pressure, either alone or in combination with other genes in the pathways, are responsible for a major part of blood pressure variation. The present findings may provide evidence that a common variant with a small effect, actually too small to influence blood pressure by itself, could be related to blood pressure variation and hypertension through combination with other common variants with a small effect allele in the same pathway. Since the effects (odds ratios) of the genes are very small, they could not be detected if their allele frequencies are low. These findings may underlie the failure of replication of candidate gene findings.

In the first case-control studies, 10 of 38 positive SNPs were found to deviate from the Hardy-Weinberg equilibrium (HWE). It has been shown that several underlying mechanisms may cause the violation of HWE (*45*). However, the first study was a screening study to pick up the possible posi-

tive SNPs, and all positive SNPs are scheduled to be genotyped in a large general population to reconfirm the findings, as five G-protein related SNPs were genotyped in the second population in the present study. Accordingly, in the present study, all results were present without any correction based on HWE.

In summary, the findings of the present study indicate that a systemic multiple candidate gene approach can be used to identify not only susceptibility genes but also susceptibility pathways in which related genes may synergistically collaborate through gene–gene interactions to predispose to hypertension. Our findings suggest that the CD/CV hypothesis can be challenged with numerous combinations of common variants with small effects.

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