information, such as the peak positions and heights, were manually extracted by the PickPeak and MultiPeaks programs, developed by Applied Biosystems Japan, from the multipeak pattern in the chromatogram ABI fsa files.

In the first stage, 95 case and 95 control subjects were subjected to association analysis using all of the 18 977 markers. Among them, markers showing statistical significance of $P<0.05$ were subjected to the second stage with another 120 case subjects and 120 control subjects. The markers showing statistical significance of $P<0.05$ in the second screening were subjected to a third stage with another 170 case subjects and 170 control subjects. All of the positive markers that remained statistically significant ($P<0.05$) in the stage 3 screening were confirmed by individual genotyping using the same set of 385 case subjects and 385 control subjects as the final step.

Marker Information

MS sequences were computationally detected from all of the chromosomes except for the Y chromosome (in 4 versions of the human genome draft sequence: Golden Path Jun 2004 to the National Center for Biotechnology Information build 35). At present, our laboratory has built 27 037 markers as a full set.[25]

In this study, we used 18 977 markers with an average spacing of 145.9 kb (Tables I and II, available online at http://hyper.ahajournals.org.) from 19 654 markers in the first built set. The other 678 markers were excluded because of problems with the PCR reaction and marker quality.

The MS markers were investigated for repeat polymorphisms in 200 healthy Japanese by using the DNA pooling method. Our criteria for selection of MS markers for the hypertension association study were dinucleotide repeats with >10 repeats; tri-, tetra-, and pentanucleotide repeats with >5 repeats; and polymorphic MS markers with heterozygosity of >30% but not those with heterozygosity of >85% to eliminate any unstable and highly mutated MS markers. We chose PCR primers that contained no SNPs in the sequences to prevent differential amplification. Seven PCR primer pairs of 54 MS markers for individual typing were redesigned to improve the efficiency of PCR (Table III). Detailed information on the 27 039 MS markers is available on the Japan Biological Information Research Center homepage (http://www.jbirc.aist.go.jp/gdbs/).

Statistical Analysis

The measurements of the heights of multiple peaks in the pooled DNA were applied to association analysis. To calculate *P* values, we used 2 types of Fisher's exact test for 2×2 contingency tables for each individual allele and 2×*m* contingency tables for each locus, where *m* refers to the number of marker alleles observed in a population. The Markov chain/Monte Carlo simulation method was used to execute Fisher's exact test for the 2×*m* contingency table. The simple "allelic" but not "genotype" association was presented for the 2×2 contingency tables for MSs. These analyses were executed using the software package, AStat. The method of Pritchard and Rosenberg[36] was used for the detection of stratification in case and control populations using 23 MS markers. The Hardy–Weinberg test for allele frequency distributions at the MS loci was performed by *P* test for differentiation, as determined by GenePop 3.4. Other basic analyses were carried out using Microsoft Excel.

The authors had full access to the data and take responsibility for its integrity.

Results

Three-Stage Screening: Pooled DNA Typing

Before the 3-stage screenings, we verified spurious associations through the method of Pritchard and Rosenberg[36] using 23 randomly selected MS markers from each of chromosomes 1 to 22 and X, with an absence of any significant stratification in either the case or control populations (data not shown). This test is important to prevent spurious associations by population stratifications, especially for late-onset diseases, such as essential hypertension.

We initially identified 54 markers as potential hypertension susceptibility loci by 3-stage screening of 3 independent case–control populations (stage 1: 95; stage 2: 120; stage 3: 170 patients with essential hypertension and normotensive healthy individuals; Table 2). Three-stage screenings were intended to sequentially replicate the results in the 3 independent sample populations and eliminate pseudopositive markers resulting from type I errors.[37,38]

The number of markers decreased from 18 977 to 1160 markers in the first screening, then to 284 markers in the second screening, and finally to 54 markers in the third screening. The significance ($P<0.05$) of the association of positive markers was assessed by the Fisher's exact test, using either 2×2 or 2×*m* (*m*=number of alleles) contingency tables. Both 2×2 and 2×*m* analysis were performed together at each marker. If either of the 2 had $P<0.05$, the marker was judged as a positive marker. Finally, 54 markers significant in all of the screening sets were significant in the 2×2 test, and some of the markers were significant in the 2×*m* test. The concordance between the 2×2 and 2×*m* tests was relatively low (stage 1: 60%; stage 2: 64%; stage 3: 81%). All of the positive markers were checked by the Hardy–Weinberg test for allele frequency distributions at the MS loci, and then significant markers ($P\leq0.05$) in the Hardy–Weinberg test were excluded. The positive rates in the second and third screenings were higher than that in the first. This might be partially because of experimental artifacts of the pooled DNA method as reported by Sham et al[39] and Shaw et al.[40]

Individual Typing

The results of pooled typing were presumptive, so we genotyped a total of 770 individuals (385 case subjects versus 385 control subjects) and reanalyzed the 54 markers in the 3-stage screening procedure. These individuals are the same individuals as used in pooled typing and were not from a new cohort. Ultimately, we reduced the number of positive markers from 54 to 19 loci by using individual genotyping in the genome-wide association study for hypertension (Table 2). All 19 of the markers were significant ($P<0.05$) by 2×2 analysis, but only 3 markers were also significant ($P<0.05$) by 2×*m* analysis. In addition, the odds ratios ranged from 0.13 to 1.8 (Table 2).

The 19 genomic loci were observed on chromosome 2, 3, 4, 6, 10, 13, 17, 18, 19, and 20 (Table 3). The observed and expected frequencies of each genotype for the 19 markers in the case and control subjects were in Hardy–Weinberg equilibrium (data not shown). In considering the LD range, the susceptibility genes for hypertension were estimated to reside in a 100- to 150-kb region from each marker. We have also provided a list of the genes that are known to be positioned closest to the centromeric and telomeric side of each marker (Table 3) to highlight the locations of the 19 positive markers. The chromosomal location of the 19 markers in our study (Table 3) was compared with those identified in previous studies (Table 4). Essentially, 3 chromosomal locations were found to overlap in comparison with other studies: chromosome locations 2p11.1-q12.3, 2p25.1, and 6q27.

TABLE 2. Summary of the Phased Genome Screen by the DNA Method and Individual Typing

	First Screening			Second Screening		Third Screening		Individual Typing	
Chromosome	No. of MS	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %
1	1516	146	10	31	21	7	23	0	0
2	1847	112	6	29	26	5	17	3	60
3	1422	66	5	15	23	3	20	3	100
4	1157	69	6	13	19	7	54	2	29
5	1173	71	6	14	20	0	0	0	0
6	1204	89	7	22	25	7	32	1	14
7	1266	66	5	18	27	2	11	0	0
8	830	58	7	12	21	0	0	0	0
9	838	32	4	6	19	3	50	0	0
10	907	45	5	11	24	2	18	1	50
11	916	40	4	10	25	0	0	0	0
12	684	25	4	6	24	0	0	0	0
13	609	32	5	11	34	2	18	2	100
14	571	29	5	6	21	1	17	0	0
15	458	34	7	9	26	2	22	0	0
16	508	25	5	9	36	0	0	0	0
17	495	42	8	17	40	5	29	4	80
18	539	27	5	8	30	2	25	1	50
19	358	33	9	8	24	3	38	1	33
20	430	23	5	7	30	3	43	1	33
21	260	15	6	2	13	0	0	0	0
22	228	16	7	5	31	0	0	0	0
X	756	64	8	15	23	0	0	0	0
Y	5	1	20	0	0	0	0	0	0
Total	18,977	1160	6	284	24	54	19	19	35

P value<0.05 was set at statistical significance by Fisher's test for the 2×2 or 2×*m* contingency table. The positive rates represented the rate of the markers, which were positive in the 2×2 or the 2×*m* analysis, to analyzed 18 977 (first), 1160 (second), and 284 markers (third).

Most of the 35 markers that were eliminated by individual typing after the 3-stage screening procedure may have been experimental artifacts or pseudopositive markers because of the DNA pooling method, PCR assay conditions, faulty peak heights during electrophoresis, PCR ghost peaks because of dissociation of labeled fluorescent reagents from a primer oligonucleotide, complications resulting from stutter, and additional nucleotide bands inherent to a particular MS.

Discussion

High-Density MS Markers

We conducted a 3-stage genome-wide scan of 3 independent case–control populations by an association test using 18 977 MS markers to identify susceptible genes for essential hypertension. In this study, we used 18 977 markers with an average spacing of 145.9 kb as the first built set (Tables I and II). Based on recent knowledge, the average length of LD between the disease-susceptible SNPs and the nearby MS alleles is ≥100 kb.[26–31] In other words, if the disease-susceptible SNPs are harbored between 2 neighboring MS markers at an interval ≤200 kb, LD between the disease-susceptible SNPs and either of the nearby MS will be proved. The use of average spacing of genetic markers across 100 to 200 kb of the entire genome is the best practical solution in genome-wide association analysis before availability of a genome-wide LD map, because the LD pattern varies between different regions of the human genome depending on several factors, such as allele frequency, mutation, and recombination.[33] Therefore, our first step for genome-wide analysis was to collect enough MS markers (>18 000 MS, 1 MS at every 150 kb) to cover the euchromatic area (≈90%) of the human genome (3 giga base; 3×10^9 kb ×0.9/150 kb=18 000). The remaining part of the genome was mostly heterochromatin restricted mainly to centromeres and telomeres, rich in repetitive sequences and believed to lack expressed genes. This 150-kb spacing of MS markers would enable us to assure an average 75-kb LD interval, which was presumed to detect the presence of disease-susceptible loci flanked by 2 neighboring MS markers across the whole genome.

Although 54 MS markers were found significant in all 3 stages of the pooling experiments, only 19 (35%) of them were confirmed to be significant when individual typing was performed. This indicates the importance of performing

TABLE 3. Nineteen Positive Microsatellite Markers From Individual Typing

Markers	Cytobands	No. of Alleles	Positive Alleles	Allele Frequencies		*P*		Odds Ratio	95% CI	Nearest Gene Name
				Case	Control	2×2	2×*m*			
HUMUT617	6q27	5	1	0.155	0.215	0.00287	0.02289	1.54	1.08 to 2.21	SMOC2
D2S0226i	2q35	16	1	0.096	0.058	0.00523	0.12651	1.73	1.18 to 2.55	XRCC5
D17S0287i	17q21.33	8	1	0.390	0.323	0.00746	0.08458	1.34	1.09 to 1.65	CROP
D17S0351i	17q24.3	10	4	0.146	0.198	0.00812	0.00229	0.69	0.53 to 0.90	KCNJ16
D19S0134i	19p13.2	28	2	0.070	0.040	0.01212	0.06104	1.80	1.14 to 2.84	ZNF358
D2S0208i	2q11.2	9	1	0.224	0.174	0.01468	0.06679	1.38	1.07 to 1.77	CHST10
D13S0183i	13q31.3	11	2	0.230	0.179	0.01545	0.13986	1.37	1.07 to 1.76	Unknown
D4S0818i	4p16.1	17	1	0.112	0.153	0.01950	0.35114	0.70	0.52 to 0.94	SORCS2
D2S0949i	2p25.1	11	2	0.105	0.070	0.02343	0.09465	1.54	1.08 to 2.21	LPIN1
D20S885	20p11.23	17	1	0.123	0.165	0.02403	0.55390	0.71	0.53 to 0.95	Unknown
D10S0517i	10q26.13	15	1	0.223	0.177	0.02542	0.10058	1.33	1.04 to 1.72	TACC2
D17S0231i	17p13.1	8	1	0.615	0.558	0.02608	0.18437	1.26	1.03 to 1.54	Unknown
D3S0865i	3p21.31	13	1	0.245	0.199	0.03162	0.27624	1.31	1.03 to 1.66	XCR1
D3S1129i	3p22.1	26	1	0.149	0.112	0.03273	0.43997	1.39	1.03 to 1.88	Unknown
D3S0046i	3p26.1	19	1	0.001	0.010	0.03862	0.59448	0.13	0.02 to 0.73	GRM7
D17S790	17q22	14	1	0.026	0.047	0.04028	0.20889	0.54	0.31 to 0.94	Unknown
D18S0390i	18q22.1	17	2	0.140	0.179	0.04227	0.11569	0.75	0.57 to 0.98	Unknown
G09023	13q33.3	8	1	0.204	0.249	0.04245	0.03990	0.78	0.61 to 0.99	Unknown
D4S0370i	4q34.4	18	1	0.052	0.078	0.04780	0.54383	0.65	0.43 to 0.98	Unknown

individual typing after all of the pooling experiments to validate the pooled frequency estimates.

Essential Hypertension Susceptibility Genes

We have identified 19 MS loci associated with essential hypertension and compared our findings with those of 6 previous large-scale genome-wide studies that are summarized in Table 4. The loci of linkage analysis in the previous 9 reports were too wide (≥5 megabases) to identify and speculate about disease susceptibility genes. Three of the 19 identified regions in our study overlapped with a region identified in other races (Table 4). The studies in Table 4, except for the admixture mapping study,[13] were linkage studies and suggest a much broader region than our results. For example, we found that the positive MS locus D2S0949i is located on cytoband 2p25.1, and this finding is in accordance with the admixture mapping results obtained by Zhu et al,[13] who found evidence for linkage with a marker on chromosome 2p25.1. This concordance between 2 different studies suggests that chromosome 2p25.1 contains an unknown candidate gene for essential hypertension. Interestingly, our MS marker is located within the LPIN1 gene sequence (NM 145693.1), and this is a candidate gene for human lipodystrophy, a disease characterized by loss of body fat, fatty liver, hypertriglyceridemia, and insulin resistance. There have been no reports to indicate that LPIN1 is a candidate gene for essential hypertension, but lipin expression is important for metabolic homeostasis.[40] In consideration that hypertension is an associated factor in the metabolic syndrome characterized by obesity, hypertriglyceridemia, and insulin resistance,[41,42] LPIN1 deserves to be studied as a new candidate gene.

Another significant MS marker, HUMUT617 on 6q27, is in the same cytoband position reported by Caulfield et al[14] Our marker was located within the SMOC2 gene sequence (NM_022138) that codes for a modular extracellular calcium-binding protein[43] and a smooth muscle–associated protein upregulated during neointima formation.[44] There have been no previous reports suggesting any connection between SMOC2

TABLE 4. Summary of Genome-Wide Scan Mapping Analyses on Blood Pressure

References	Chromosome	Ethnicity
Zhu et al.[13]	2p25.1, 3q13.31-33, 6q24, 21q21	Admixture
Caulfield et al.[14]	2q24.1, 5q13.1, 6q27, 9q34.11	White
Rao et al.[15]	2p	Blacks and whites
Kardia et al.[16]	No evidence	Blacks and non-Hispanic whites
Thiel et al.[17]	1	Blacks and whites
Ranade et al.[18]	10p	Chinese and Japanese origin
Harrap et al.[19]	1p34.3-1p31, 4q21-28, 16p13.1-16p12, Xp11.4-Xq11	White
Atwood et al.[20]	2p11.2, 2q12.2, 8q24.3, 18q23, 21q22.13	Mexican Americans
Rice et al.[21]	1p22.3-p13.1, 2p11.1-q12.3, 3q13.31-q26.32, 5p15.2-p12, 7q32.1-q36.1, 8q21.11, 10p14, 12p13.33, 14q11.2-q12, 19p13.3, 22q13.1-q13.2	White

Overlapped locations mapped by this study in comparison with previous studies are underlined.

and BP, but this gene may be involved in the progression of atherosclerosis in the aorta.[44]

Perspectives

We performed an association analysis of essential hypertension using a high-density set of polymorphic MS markers with original, multistep methodology. The outcome was a rapid and efficient path to detect genomic susceptibility loci for a highly complex disorder. MS markers basically play a role as location markers for regions containing susceptibility and protective genes. Rarely, MS markers may be the causative variance themselves. The next step is to identify susceptibility and protective genes in the 19 narrow regions by SNP, LD block, and haplotype analysis. It is also important to replicate these results in different subjects, ethnic groups, and a larger number of samples. The future successful accomplishment of such analysis will also open the door to investigating the etiology of other multifactorial disorders, including common diseases such as bronchial asthma, type 2 diabetes mellitus, obesity, arteriosclerosis, schizophrenia, and psoriasis.

BP is influenced by nongenetic factors, such as salt intake. In the present study, because we did not focus on salt-induced hypertension, the amounts of urinary excretion of sodium were not examined. It might be noteworthy to perform studies specializing in genes related to salt-induced hypertension.

Acknowledgments

We thank Tomoko Shiota, Ritsuko Nishizaki, Eriko Tokubo, Mikiko Hirayama, Asumi Takaki, and Takashi Shinomiya for their technical assistance. We also thank all of the staff and doctors who contributed to blood sample collection from the subjects.

Source of Funding

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas and "Medical Genome Science" from the Ministry of Education, Culture, Sports, Science and Technology.

Disclosures

None.

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Endocr (2007) 31:149–153
DOI 10.1007/s12020-007-0024-9

ORIGINAL PAPER

Two novel genotypes of the thiazide-sensitive Na-Cl cotransporter (*SLC12A3*) gene in patients with Gitelman's syndrome

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Received: 5 March 2007 / Accepted: 14 May 2007 / Published online: 15 June 2007

Abstract Gitelman's syndrome is an autosomal recessive disorder marked by salt wasting and hypokalaemia resulting from loss-of-function mutations in the *SLC12A3* gene that codes for the thiazide-sensitive Na-Cl cotransporter. Gitelman's syndrome is usually distinguished from Bartter's syndrome by the presence of both hypomagnesaemia and hypocalciuria. Although recent advances in molecular genetics may make it possible to both diagnose and differentiate these diseases, the phenotypes sometimes overlap. Here we report two sporadic cases of Gitelman's syndrome and two novel genotypes of *SLC12A3*. Patient 1 was a compound heterozygote with a known missense mutation, L849H, and a novel mutation, R852H in exon 22. Patient 2 was homozygous for the missense mutation L849H. To our knowledge, this is the first report of a patient homozygous for 849H. Interestingly, both patients were affected with autoimmune thyroid disease. Patient 1 was affected with Hashimoto's disease, and Patient 2 was affected with Graves' disease. The symptoms of Patient 2 were more serious than those of Patient 1. Although the patients both carried the 849H allele (Patient 1 as a heterozygote and Patient 2 as a homozygous), their clinical symptoms differed. The difference in the clinical features may have been due both to phenotypic differences and the fact that Gitelman's syndrome is a complicated disorder.

Keywords Gitelman's syndrome · Missense mutation · Compound heterozygote · Homozygous · Graves' disease

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Introduction

Gitelman's syndrome (GS) was first described by Gitelman and colleagues in 1966 [1]. This syndrome has characteristics similar to those of Bartter's syndrome, such as salt wasting, hypokalaemia, metabolic alkalosis and normal blood pressure under hyperreninaemic hyperaldosteronism. GS is distinguished from Bartter's syndrome by the presence of hypomagnesaemia and hypocalciuria. However, these features also present in a subset of Bartter's syndrome patients [2–4]. And current pivotal treatments are potassium replacement for both syndromes. Therefore, there may be sufficient overlap between the two syndromes from clinical viewpoints. Advances in molecular genetics have clarified that GS are caused by renal tubule electrolyte transporter dysfunction. GS is an autosomal recessive disorder resulting from mutations in the gene (*SLC12A3*) encoding the thiazide-sensitive Na-Cl cotransporter (TSC) [5–8]. The human *SLC12A3* gene, which is located on chromosome 16, consists of 26 exons and encodes a protein that contains 12 putative transmembrane domains with long intracellular amino and carboxy termini.

To date, a number of different *SLC12A3* mutations have been reported, including missense, frame shift, nonsense,

and splice-site mutations. Missense mutations are the most common presented abnormality. The majority of these mutations have been collected in the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/). In a recent study, the functional consequences of GS mutations were assessed by measuring tracer $^{22}Na+$ uptake in Xenopus oocytes or in Chinese hamster ovary (CHO) cells [9, 10]. The T180K variation was found to be simply a polymorphism, whereas the L849H mutation was found to be a loss-of-function mutation that appears to be responsible for GS [10].

It is known that thyroid status affects electrolyte balance in the kidney. Disturbances of thyroid function are accompanied by widespread alterations in renal haemodynamics and tubular handling of electrolytes [11, 12]. Munro et al. [13], studying exchangeable potassium and sodium, found that PTU or surgical therapy of hyperthyroidism caused an increase in body potassium but no consistent change in sodium content, and therapy of myxoedema with T_4 caused a loss of both sodium and potassium. Patients with thyroid disease may tend to develop the symptoms of GS. In this report, we describe two sporadic cases of Gitelman's syndrome whose patients were affected with autoimmune thyroid disease and two novel genotypes of *SLC12A3*.

Patients and methods

Case 1

Patient 1, a 40-year-old woman, was referred to our department for evaluation of hypokalaemia. She had complained of shoulder discomfort and numbness, and painful muscle cramps in her extremities. She had been treated for diabetes mellitus for several years. No other family member had a similar illness, and there was no history of parental consanguinity. The physical examinations were unremarkable except for a homogenous goiter. Her blood pressure was 136/86 mmHg, and her resting heart rate was 78 beats/min. Her body mass index (BMI) was 26.3 kg/m^2. She had neither muscle weakness nor paresthesia. The deep tendon reflexes were normal. The biochemical studies showed moderate hypokalaemia, normomagnesaemia and normotensive hyperreninaemia. Urinary calcium to creatinine ratio was 0.029. Arterial blood gas showed mild metabolic alkalosis. Thyroid function was normal, although anti-thyroperoxidase antibodies were present (Table 1). Due to these clinical and biochemical findings, we concluded that this was probably a case of GS and also diagnosed Hashimoto's disease. We performed genetic analyses of *SLC12A3* to confirm the diagnosis of GS.

Table 1 Clinical and biochemical findings in two patients with Gitelman's syndrome

Clinical symptoms	Normal range	Patient 1 Shoulder discomfort numbness	Patient 2 Periodic paralysis thirst, diuresis
Serum electrolyte levels			
Na	136–148 mmol/l	141	144
K	3.6–5.0 mmol/l	3.3	1.7
Cl	98–109 mmol/l	98	96
Ca	8.8–10.8 mg/dl	9.5	8.9
Mg	1.7–2.3 mg/dl	1.8	1.5
Urinary electrolyte excretions			
Na	130–350 mEq/day	67.2	145.6
K	40–65 mEq/day	41.8	90
Urinary Ca/Cr	0.05–0.15	0.029	0.02
Urinary NAG	1.3–4.5 U/day	0.6	9.1
Urinary β2MG	5–300 μg/l	13	70
Plasma aldosterone	29.9–159 pg/ml	130	79
Plasma renin activity	0.3–2.9 ng/ml/h	13	22
TSH	0.34–3.80 μU/ml	2.02	0.01
Free T_3	2.0–3.8 pg/ml	3.66	24.58
Free T_4	0.8–1.5 ng/dl	1.41	>7.77
TPO-Ab	<0.3 U/ml	124	–
TRAb	<1.0 IU/l	–	17.9
BGA (room air)			
pH	7.35–7.45	7.458	7.506
HCO_3^-	22–26 mmol/l	29.3	35.4
BE	−2 to +2 mmol/l	5.2	12.2

NAG, N-acetyl-β-D-glucosaminidase; β2MG, β2-microglobulin; TSH, thyroid stimulating hormone; T_3, triiodothyronine; T_4, thyroxine; TPO-Ab, antithyroid peroxydase antibody; TRAb, TSH receptor antibody; BGA, Blood gas analysis; BE, Base excess

Case 2

Patient 2 was 28-year-old woman. For 1 year, she had suffered from transient paralysis of the lower extremities whenever she ran a fever. She was admitted to our hospital because of muscular weakness that progressed to paralysis involving all extremities. She also had complained of thirst and excessive urination. Relevant medical history included a diet at the age of 22 years that led to hospitalization with tetany due to hypocalcaemia at 23 years of age. She denied any form of self-medication, surreptitious diuretic and laxative abuse or persistent vomiting and diarrhoea at present. Her parents were not consanguineous. Her mother and a maternal grandmother had a history of hyperthyroidism. On physical examination, her blood pressure was 118/65 mmHg, her resting heart rate was 108 beats/min and BMI was 27.4 kg/m^2. The thyroid gland was enlarged.

Manual Muscle Testing [14] revealed grade 4 in her upper limbs and grade 2 in her lower limbs. The deep tendon reflex was present but decreased. Laboratory tests showed severe hypokalaemia, low thyroid stimulating hormone (TSH) levels, high free T_4 levels and the presence of anti-TSH receptor antibodies, which led to the diagnosis of Graves' disease. Furthermore, she had hypomagnesaemia, hypocalciuria and normocalcaemia. The plasma aldosterone concentration was normal, although plasma renin activity was elevated. The molar ratio of urinary calcium/creatinine was 0.02. Arterial blood gas showed metabolic alkalosis (Table 1). On the basis of these findings, the diagnosis of GS was made. Genetic analysis of the *SLC12A3* gene was performed to confirm diagnosis.

Mutation analysis

We performed *SLC12A3* gene mutation analysis for each patient. We also had the opportunity to study the parents of Patient 1. They did not have any apparent clinical symptoms. Informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee of Nihon University. Blood samples were collected into tubes containing 50 mM EDTA-2Na, and genomic DNAs were extracted as described [15]. We positioned primers in introns at the 5′ and 3′ boundaries of *SLC12A3* to amplify exons for sequence analysis (Table 2). Exon 1 was amplified with two PCR reactions that produced two overlapping PCR products. PCR products were purified with ExoSAP-IT® reagent (GE Healthcare Life Sciences, Piscataway, NJ) including exonuclease I (Exo I) and shrimp alkaline phosphatase (SAP) and subjected to automated DNA sequencing analyses with fluorescence-labelled dideoxyterminators (BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (ABI PRISM 3700 Genetic Analyzer, Applied Biosystems) [16]. Sequencing was performed for both strands. Patient 1 was found to be a compound heterozygote with a single-base substitution at nucleotide 2552 (CTC-to-CAC, L849H) and a substitution at nucleotide 2561 (CGC-to-CAC, R852H) in

Table 2 Primers used for sequencing analysis of the *SLC12A3* gene

Target regions	Forward primers	Reverse primers
Exon1–1	5′-GATCCTGGCCCCTCCCTG	5′-TGCTGTTGGCATAGTGCTCA
Exon1–2	5′-CCACCAGCTGCCTATGACA	5′-CGAGGTCACACAGCAGGAAG
Exon2	5′-GAGACGCCGTCCCTAGCACC	5′-TGGACATCACGCACCACCCA
Exon3	5′-GGTGTCACCCAGGTGGCCTC	5′-GGCAAGCTGGGAAGAATGGG
Exon4	5′-GGCTCCTCCCTTGGGAAATG	5′-GACCCACGAGAGGAGGGCCT
Exon5	5′-ACCGACTCATCTGGTTTCAT	5′-GATCCCTCTACCCAGGGTCC
Exon6	5′-GGTGTTGCAGCCTGGCCCAT	5′-ACGTGACCACCTCCATGTCC
Exon7	5′-GGCTTCCCAGAGAGGTAGAA	5′-GTCCCCAGAGCCATGGTCAG
Exon8	5′-GGTCAAGCCCTCCAGGTGAG	5′-TAGCCCCTGTGCAGTGCCAG
Exon9	5′-CCTTCAGGACCCTGCTAT	5′-GACACTGCAGGGTGGAGGCC
Exon 10	5′-CAGAGTAAGGAGGGAAGGCA	5′-CCACTGTGTCTGGTGGGTCA
Exon 11	5′-CAGCCCTCACCGTGGAGTCC	5′-CCCACCCCCTGTCATCTCGA
Exon 12	5′-GGAAGTGGCAGGTCCCAGCC	5′-CAGGAGGCCAGGCCCTGTGA
Exon 13	5′-AGTTGCCCAACAGGCTGTCC	5′-CCATGCCCCAGTTCCTCCTG
Exon 14	5′-CGACTGCCAGGCATGCCCAC	5′-CCGCCTGCATGGCTACCCTG
Exon 15	5′-CTGGTTTCCTCTAGTGATTC	5′-TCACTGGCCCTGGGGTCCCA
Exon 16	5′-CTCTCCTGATGGCTCCTGCC	5′-TGCTGGGTTTACAGGCATGAG
Exon 17	5′-GAGGGTGAAGGCAGCTGGTG	5′-GCCACCAAGCCGTAAGTCCT
Exon 18	5′-GATCACCAACTCTGCCCCTC	5′-ATGGCCCAAATTAACAGACC
Exon 19	5′-AGTGGGAGCTGGGGGAGAAG	5′-CTAGAACTTTCTGGGAGTGG
Exon 20	5′-ACGGTGCCCTCAGACAAGGAG	5′-GAGTGCCCTGAGCTCTGAGTG
Exon 21	5′-GCGCGGCGCTGGCTCTGC	5′-CCGGGCAGGAGGGCTGATCC
Exon 22	5′-ATTCTTGTCATGACTCACGG	5′-TGGAGCTAAGATGACACTGG
Exon 23	5′-CAGAGCAAGACGCTGTCTCA	5′-TCTCCAGGCACACAGTTGGC
Exon 24	5′-CTCAGCCGGCCTCAACCCAC	5′-CCCTGACCCAGTGATGTGTC
Exon 25	5′-GGTGAAGGATTGAGTGACCT	5′-CACCTGACTCTGGACAGACT
Exon 26	5′-CTTTGCCCATAGGGAGGAAG	5′-GAGCTGTGGACAGGGATGTC

exon 22. Familial linkage analyse confirmed that 849H was the paternal allele and 852H was the maternal allele. Patient 2 was homozygous for the L849H mutationin (Fig. 1).

Clinical course

Patient 1 was treated with potassium supplementation (potassium chloride 32 mEq/day). After 2 years of follow-up, her neuromuscular symptoms were almost absent. Patient 2 was treated with thiamazole 30 mg/day for Graves' disease and potassium supplementation (same dose as Patient 1) for Gitelman's syndrome. At 1 year of follow-up, her thyroid function was normal, and when potassium supplementation was ceased, the neuromuscular symptoms did not reappear. Although hypocalciuria persisted in both patients, serum electrolytes (magnesium and potassium) were normal.

Discussion

We describe here two cases of normotensive hypokalaemia associated with GS. This diagnosis was confirmed by genetic analysis of these patients. This is the first report of the R852H variant of *SLC12A3* and the first report of a patient homozygous for the 849H allele. Lemmink et al. [17] reported that most *SLC12A3* gene mutations are localized in the intracellular carboxy-terminal domain of the TSC protein. The L849H and R852H mutations are located in the carboxyl-terminal cytoplasmic region of the TSC protein. The L849H mutation was found to underlie GS in three different studies of Japanese patients [18–20]. Moreover, Naraba et al. [10] investigated the functional consequences of *SLC12A3* mutations by measuring tracer

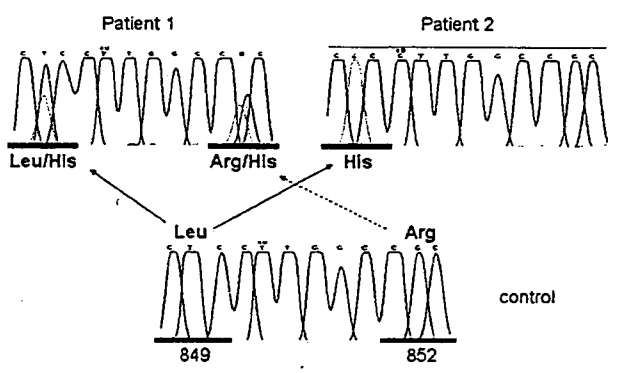

Fig. 1 Sequence analysis of the *SLC12A3* gene. In Patient 1, we found a heterozygous transition (T-to-A) at nucleotide 2552 in exon 22, resulting in a Leu-to-His substitution at amino acid 849, and a heterozygous transition (G-to-A) at nucleotide 2561 in exon 22, resulting in an Arg-to-His substitution at amino acid 852. Patient 2 is homozygous for the T-to-A transition in exon 22

^{22}Na+ uptake in CHO cells. They found that the L849H mutation is a loss-of-function mutation. We did not assess renal clearance of chloride, but Naraba et al. [10] indicated that these two cases are GS powerfully.

In contrast to Bartter's syndrome, GS is usually found in older children and young adults during routine investigation because the patients generally have mild symptoms, including cramps and fatigue, at presentation. However, in the present study, Patient 2, who had Graves' disease, had suffered from hypokalaemic periodic paralysis. Thyrotoxic periodic paralysis occurs mainly in Asian men [21, 22]. For a paralytic symptom, GS may be failed to notice as hypokalaemic periodic paralysis, such as thyrotoxic periodic paralysis or familial periodic paralysis, due to acute shift of K^+ into cells. Fortunately, Patient 2 had the typical clinical characteristics of GS, including hypokalaemia, hypocalciuria, hypomagnesaemia and increased renin levels.

It has been reported that the increased electrolyte excretion in the kidney due to excess thyroid hormone may have exacerbated the clinical features of GS [12]. This is because hypokalaemia and hypomagnesaemia were not observed after treatment of hyperthyroidism in Patient 2. It is possible that the symptoms of GS appear prominent when exposed to pathophysiologocal force to induce electrolyte imbalance such as hyperthyroidism. Symptoms of Patient 1 was mild because she diagnosed with Hashimoto's disease, but her thyroid function was normal.

Several reports showed that hypokalaemic nephropathy caused by long-term hypokalaemia could cause end-stage renal failure [23–25]. Patient 2 showed increase of urinary NAG, and we supposed she had tubular disorder. To prevent hypokalaemia-induced nephropathy, aggressive correction of hypokalaemia should be attempted.

Our patients with GS did not always have hypomagnesaemia. According to Maki et al. [20], two of seven patients with GS did not have hypomagnesaemia. Lin et al. [26] reported that four of 20 patients with GS did not have hypomagnesaemia. Recently, genetic studies have identified "transient receptor potential (melastatin) 6" (TRPM6) as the first component involved directly in epithelial magnesium reabsorption [27, 28]. Nijenhuis et al. [29] demonstrated that TSC inactivation was possibly associated with *Trpm6* downregulation. However, unknown magnesium regulators may compensate renal magnesium wasting caused by TSC defect.

In a recent study from Japan, genetic analysis revealed that the overall frequency of heterozygous GS mutations was 3.21%, which was higher than expected [30]. Confirmation of a diagnosis of GS with PCR/sequencing of *SLC12A3* is a useful tool for patients with hypokalaemia. And this genetic diagnosis of GS which precisely distinguishes GS from Bartter's syndrome may contribute to prove the disease prognosis and to develop appropriate therapy.

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© Med Sci Monit, 2007; 13(5): BR112-118
PMID: 17476189

www.MEDSCIMONIT.COM
Basic Research

Received: 2007.01.19
Accepted: 2007.03.01
Published: 2007.05.04

Postprandial plasma lipid levels are influenced by the interaction of functional polymorphisms in the microsome triglyceride transfer protein and β3 adrenergic receptor genes

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Source of support: Departmental sources

Summary

Background: The effects of polymorphisms in the genes encoding microsome triglycerides transfer protein (MTP) and β3-adrenergic receptor (β3-AR) on lipid and glucose metabolism were investigated.

Material/Methods: Clinical phenotypes related to lipid and glucose metabolism were evaluated during dietary loading (17 g of fat, 750 Cal) and glucose loading (75 g glucose). MTP and β3-AR genotypes were determined by restriction fragment length polymorphism.

Results: Subjects with the Arg64 β3-AR gene (Arg+) polymorphism showed significantly higher fasting (FTG) and postprandial (PTG) triglyceride levels, fasting plasma glucose (FPG), fasting plasma immuno-reactive insulin (FIRI) and HOMA-R in comparison with Trp64 homozygotes. Subjects with the T allele (T +) of MTP –164T/G polymorphism (with T allele) showed significantly lower levels of FPG, FIRI, HOMA-R and PTG than did subjects without the T allele (T–). To evaluate the interaction of the polymorphisms, we divided our subjects into four groups. T–/Arg–, T–/Arg+, T+/Arg– and T+/Arg+. In these four groups, only T–/Arg+ showed significantly higher PTG levels. Plasma glucose levels were significantly higher at 60 and 120 min after oral glucose loading in in the T–/Arg+ subjects.

Conclusions: In this study, we identified an example of genotypic interactions that influence the clinical phenotype in multi-factorial diseases.

key words: **postprandial hyperlipidemia • microsome triglyceride transfer protein • β3-adrenergic receptor • glucose intolerance • hypertriglyceridemia**

Full-text PDF: http://www.medscimonit.com/fulltxt.php?IDMAN=10064

Word count: 1965
Tables: 2
Figures: 4
References: 52

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BACKGROUND

Several genetic forms of hyperlipidemia are results of defects in lipoprotein receptors, apolipoproteins, and enzymes of lipid metabolism; however, the mechanisms underlying most forms of primary hyperlipidemia are unknown.

Familial combined hyperlipidemia (FCHL) was originally described in the early 1970's [1]. Affected members of FCHL families present with different lipid phenotypes: hypercholesterolemia, hypertriglyceridemia or combined hyperlipidemia and high serum apolipoprotein B [2]. FCHL is also known to share features of metabolic syndrome, which include hyperlipidemia, insulin resistance, obesity and hypertension, in higher prevalence compared with that of the general population [3–4]. Therefore, individuals with FCHL appear to comprise a subset of the metabolic syndrome.

Plasma lipid levels in FCHL individual are determined by a complex interaction of physiological processes influenced by major and minor genes. The distinct lipid phenotype observed in one pedigree appears to be the result of an altered response to a major gene due to mutation of minor genes [5–7].

Several studies have suggested that a heritable component determines the magnitude of postprandial hyperlipidemia [8]. Genes reported to affect postprandial lipid levels, including lipoprotein lipase (LPL) [9,10], hepatic lipase (HL) [11], fatty acid binding protein 2 (FABP2) [12] and microsome triglyceride transfer protein (MTP) [13], have been suggested as candidates for FCHL. Other metabolic characteristics of FCHL include impaired chylomicron remnant clearance [13], disturbed postprandial free fatty acid (FFA) metabolism [14] and impaired FFA uptake by fibroblasts and adipocytes [15]. In a recent report, postprandial changes in apolipoprotein B100 and apolipoprotein B48 were described by Karpe et al. [15].

We previously described a locus affecting plasma triglycerides levels in a rat model of FCHL. The region containing this locus is syntenic with the region containing the human quantitative trait locus (QTL) for small dense LDL concentration. The MTP gene is located in the middle of this region and is a possible candidate gene for FCHL [16]. MTP plays a role in formation of VLDL in the liver and chylomicrons in the intestine by transferring core lipids to the apoB molecule. A functional polymorphism in the promoter region of the MTP affects promoter activity *in vitro* [17]; therefore, we hypothesized that the functional polymorphism in the promoter of the MTP gene alters the response of the promoter to metabolic stimuli resulting from other genetic alterations.

β3-adrenergic receptor (β3-AR) is expressed predominantly in fat and adipocytes lining the gastrointestinal tract [18]. The primary role of β3-AR is thought to be regulation of the resting metabolic rate and lipolysis [19]. The Trp64Arg polymorphism in β3-AR is closely associated with major phenotypes of metabolic syndrome, including abdominal obesity [20] and insulin resistance [21,22].

To find one of the factors determining the response against metabolic effect induced by the other genetic variation, we examined the interaction of MTP and β3-AR gene mutations on postprandial plasma lipid and glucose levels.

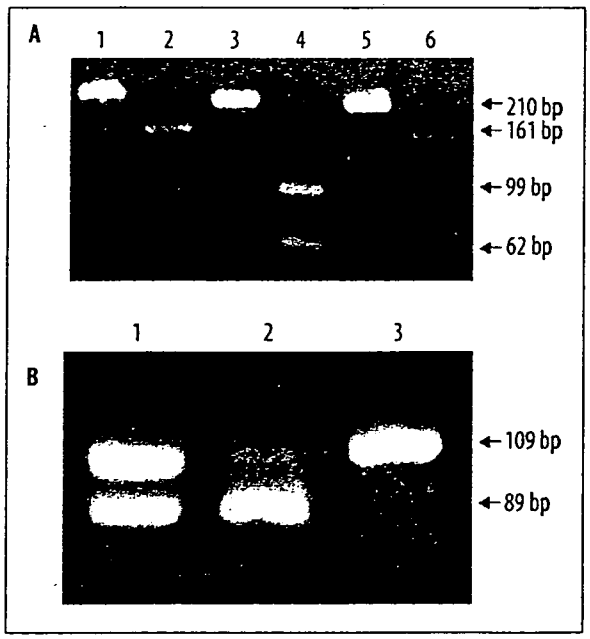

Figure 1. Detection of polymorphism of the β3AR and MTP gene by PCR and restriction fragment length polymorphism. A The PCR products(210 bp) were digested with the restriction enzyme BstOI. Lane 1,3,5 shows a PCR product; lane 2, an Arg64 homozygote; lane 4 Trp64 homozygote; and lane 6, Trp64/Arg64 heterozygote. B PCR products (109 bp) were digested with the restriction enzyme HphI. Lane 1 shows –493G/T heterozygote; lane 2–493G homozygote; and lane 3, –493T homozygote.

MATERIAL AND METHODS

Subjects

The study population included 13 healthy carriers of the –493G/T polymorphism in the MTP gene and /or 14 variants of the Trp64Arg polymorphism in the β3-AR gene. Subjects were through the health check-up program of Nihon University Itabashi Hospital. All carriers and control subjects were healthy Japanese aged 42 to 60 years and each subject was interviewed regarding lifestyle factors. None of the subjects consumed a special diet, and the amounts of physical activity and alcohol consumption were recorded. The presence of thyroid, renal or liver diseases was excluded with routine laboratory tests. The study protocol was approved by the Ethics Committee of the Second Department of Medicine, Nihon University School of Medicine.

Genotyping

Peripheral blood samples were collected after informed consent was obtained from each subject. Genomic DNA was isolated from leukocytes by standard methods. A mismatch PCR approach developed by Karpe et al. [17] was used to genotype the –493G/T polymorphism in the MTP gene promoter. A mutation in the 5' primer used for PCR of a geneproduct including the –493 site gave rise to an HphI cutting site for the –493G allele. Genotyping of the

Table 1. Plasma lipid and glucose metabolism-related phenotypes in subjects classified according to MTP or β3AR polymorphism.

	β3AR		MTP	
	Trp/Trp	Arg/Arg+Arg/Trp	G/G	G/T+T/T
n	24	13	23	14
BMI	23.7±3.3	25.2±1.0	24.5±0.6	23.5±1.1
TG (before loading) (mg/dl)	112.4±10.6	148.9±15.0*	129.4±9.7	111.8±18.6
TG (after loading) (mg/dl)	157.9±15.0	241.2±36.8*	203.5±20.9	144.2±22.5**
delta TG	45.6±7.6	92.3±23.1	74.1±12.8	32.4±7.0**
TC (before loading) (mg/dl)	197.9±7.6	186.2±9.5	195.7±8.2	192.1±8.5
TC (after loading) (mg/dl)	201.8±7.8	194.8±9.8	202.7±8.6	194.3±7.8
delta TC	3.9±2.0	8.6±3.1	7.0±2.4	2.2±1.8
HDL (before loading) (mg/dl)	53.2±2.6	45.4±3.7	49.2±2.4	54.0±4.3
HDL (after loading) (mg/dl)	52.9±2.8	43.4±3.7	48.3±2.8	53.2±4.1
delta HDL	−0.3±0.7	−2.0±1.6	−0.8±1.0	−0.8±0.7
RLP (before loading) (mg/dl)	4.3±0.5	4.9±0.5	4.6±0.3	4.4±0.9
RLP (after loading) (mg/dl)	7.2±0.9	10.0±1.7	9.0±1.0	6.3±1.4**
delta RLP	2.9±0.6	5.1±1.4	4.4±0.9	2.0±0.6
fasting plasma glucose (mg/dl)	92.6±1.9	102.9±3.0*	97.9±2.2	90.4±2.1**
fasting IRI (μg/ml)	5.6±1.7	8.7±1.5*	7.8±1.7	3.5±0.5**
HOMA-R	1.2±0.4	2.3±0.5*	1.8±0.4	0.7±0.1**
HbA1c (%)	5.2±0.08	5.5±0.3	5.3±0.1	5.0±0.1

* $p<0.05$ vs Trp/Trp, ** $p < 0.05$ vs G/G.
MTP – microsome triglyceride transfer protein; β3AR – β3 adrenergic receptor; BMI – body mass index; TG – triglycerides; TC – total cholesterol; HDL – high density lipoprotein; RLP – remnant like particle, IRI – immunoreactive insulin; HOMA – homeostasis model assessment; HbA1c – hemoglobin A1c.

Trp64Arg polymorphism in the β3-AR gene was performed as described previously [21].

Blood sampling

All subjects were admitted to the Nihon University Itabashi Hospital. The study was performed at 1:00 PM after a 12–16 h overnight fast. Each subject then consumed a meal that contained 17 g of fat (750 cal). After the meal, subjects were allowed to drink water until the last sample was collected. Blood samples were drawn before and 6 h after the meal.

Glucose tolerance was assessed by administration of 75 g of glucose orally after an overnight fast. Venous blood samples were drawn 10 min before glucose ingestion, at the time of ingestion, and 30, 60 and 120 min after ingestion to determine blood glucose and serum insulin concentrations. To estimate insulin sensitivity, we calculated the homeostasis model assessment (HOMA-R index (glucose × insulin)/409).

Statistical analyses

All values are expressed as mean ± standard error (SE) of the mean. Comparison of biochemical traits between genotype groups were made by analysis of variance (ANOVA) with the Scheffe post hoc test and Mann-Whitney *U* test. Values of $p<0.05$ were marked by asterisk. Statview 5.0 for Macintosh was used for statistical analysis.

Results

Digestion of 210bp PCR product with BstOI produced fragments of the following sizes: 161, 30, 12, and 7bp in Arg64 homozygotes; 99, 62, 30, 12, and 7bp in Trp64 homozygotes; and 161, 99, 62, 30, 12, and 7bp in Trp64/Arg64 heterozygotes (Figure 1A).

Digestion of 109bp PCR product with HpI produced fragments of the following sizes; 109, 89, and 20bp in -493G/T heterozygotes; 89, and 20bp in the homozygotes for the G variant; and 109bp in the homozygotes for the T variant.

The physical and biochemical characteristics of the subjects according to β3-AR and MTP genotypes are shown in Table 1. When the subjects were classified into groups according to the Trp64Arg polymorphism of the β3-AR gene (Arg+) or (Arg–), fasting and postprandial triglycerides, fasting plasma glucose, fasting IRI and HOMA-R levels were significantly higher in the Arg+ group than in the Trp homozgotes. These results are consistent with those of a re-

Table 2. Plasma lipid and glucose metabolism-related phenotypes in subjects classified according to MTP and β3AR polymorphism.

	T–/Arg–	T–/Arg+	T+/Arg–	T+/Arg+
n	15	8	9	5
BMI	23.5±0.7	26.5±0.7*	24.0±1.3	22.0±2.1
TG (before loading) (mg/dl)	112.6±9.4	163.0±17.6*	111.9±24.1	111.3±16.1
TG (after loading) (mg/dl)	165.5±17.6	279.6±42.7*	145.8±28.9	138.7±24.8
delta TG	52.9±10.8	116.6±26.9*	33.9±8.7	27.3±10.8
TC (before loading) (mg/dl)	199.0±10.8	189.1±12.1	196.1±10.2	178.3±15.1
TC (after loading) (mg/dl)	203.9±11.5	200.1±12.6	198.4±9.4	180.7±12.2
delta TC	4.9±2.9	11.0±3.8	2.2±2.2	2.3±3.9
HDL (before loading) (mg/dl)	53.6±2.5	40.5±3.8	52.7±5.5	58.3±3.2
HDL (after loading) (mg/dl)	53.4±2.5	38.3±3.4	52.1±5.3	57.0±2.3
delta HDL	-0.1±1.1	−2.3±2.2	-0.6±0.9	−0.8±0.7
RLP (before loading) (mg/dl)	4.2±0.3	5.2±0.7	4.5±1.2	3.9±0.2
RLP (after loading) (mg/dl)	7.5±1.1	11.8±2.0	6.7±1.8	5.1±0.4
delta RLP	3.3±0.9	6.6±1.7	2.2±0.7	1.2±0.6
fasting plasma glucose (mg/dl)	94.3±2.5	104.6±3.5*	88.9±2.2	96.0±3.0
fasting IRI (μg/ml)	6.9±2.5	9.4±1.8*	2.8±0.4	6.0±0.4**
HOMA-R	1.5±0.5	2.5±0.6*	0.6±0.1	1.4±0.1**
HbA1c (%)	5.2±0.1	5.6±0.3	5.1±0.1	4.8±0.4

* $p<0.05$ vs T–/Arg–; ** $p<0.05$ vs T+/Arg–.
MTP – microsome triglyceride transfer protein, β3AR – β3 adrenergic receptor; BMI – body mass index; TG – triglycerides; TC – total cholesterol; HDL – high density lipoprotein; RLP – remnant like particle; IRI – immunoreactive insulin; HOMA – homeostasis model assessment; HbA1c – hemoglobin A1c.

cent report of a Japanese population [23]. Interestingly, there was no significant difference in BMI or postprandial lipid levels between the two groups.

The -164 polymorphism in the MTP gene lies in a putative consensus sequence that is homologous to the human LDL receptor promoter sterol responsive element. We divided the subjects into groups on the basis of this polymorphism (with (T+) or without T allele (T–)). T+ subjects showed significantly lower fasting plasma glucose (90.4±2.1mg/dl), fasting IRI (3.5±0.5 μg/ml), HOMA-R(6.7±0.1) and postprandial TG (144.22±22.5mg/dl) and RLP levels (6.3±1.4mg/dl) than did T– subjects (Table 2).

To evaluate the genetic interaction between the MTP and β3AR genes, subjects were divided into four groups on the basis of genotypes: T–/Arg–, T–/Arg+, T+/Arg- and T+/Arg+. Only T–/Arg+ subjects showed significant increases in plasma triglyceride levels before (163.0±17.6mg/dl) and after dietary loading (279.6±42.7mg/dl). Plasma RLP levels also increased after dietary loading; however, the change was not statistically significant (Figure 2). In this group, plasma glucose levels were increased significantly at 60 and 120 min after oral glucose loading. There were no significant differences in the groups for plasma IRI before and at 30 min after glucose loading (Figure 3).

Because β3AR influences obesity and insulin resistance [19–21], we evaluated the effect of BMI and HOMA-R on MTP genotype. Both BMI and HOMA-R had positive effects on plasma triglyceride levels before and after dietary loading, but these influences were just additive effect for the impact of MTP genotypes on plasma triglyceride levels (Figure 4).

Discussion

Hypertension, hyperlipidemia, obesity, insulin resistance and prothrombotic and proinflammatory states are important targets of risk-reduction therapies for coronary artery disease (CAD) [24]. Human FCHL increases the risk for CAD, yields diverse lipid abnormalities within a single family, and is sometimes, associated with insulin resistance and hypertension [2,4]. Subset of FCHL patients are diagnosed as having metabolic syndrome because of similarities in the clinical features. The search for causal genes for metabolic syndrome in humans has been difficult because of its complex, heterogeneous and multifactorial nature that result from the interplay of genetic and environmental factors. Several animal strains have been described as models of human metabolic syndrome or FCHL, and several loci for metabolic phenotypes have been proposed [25–28] . We previously reported a QTL for plasma triglyceride levels in

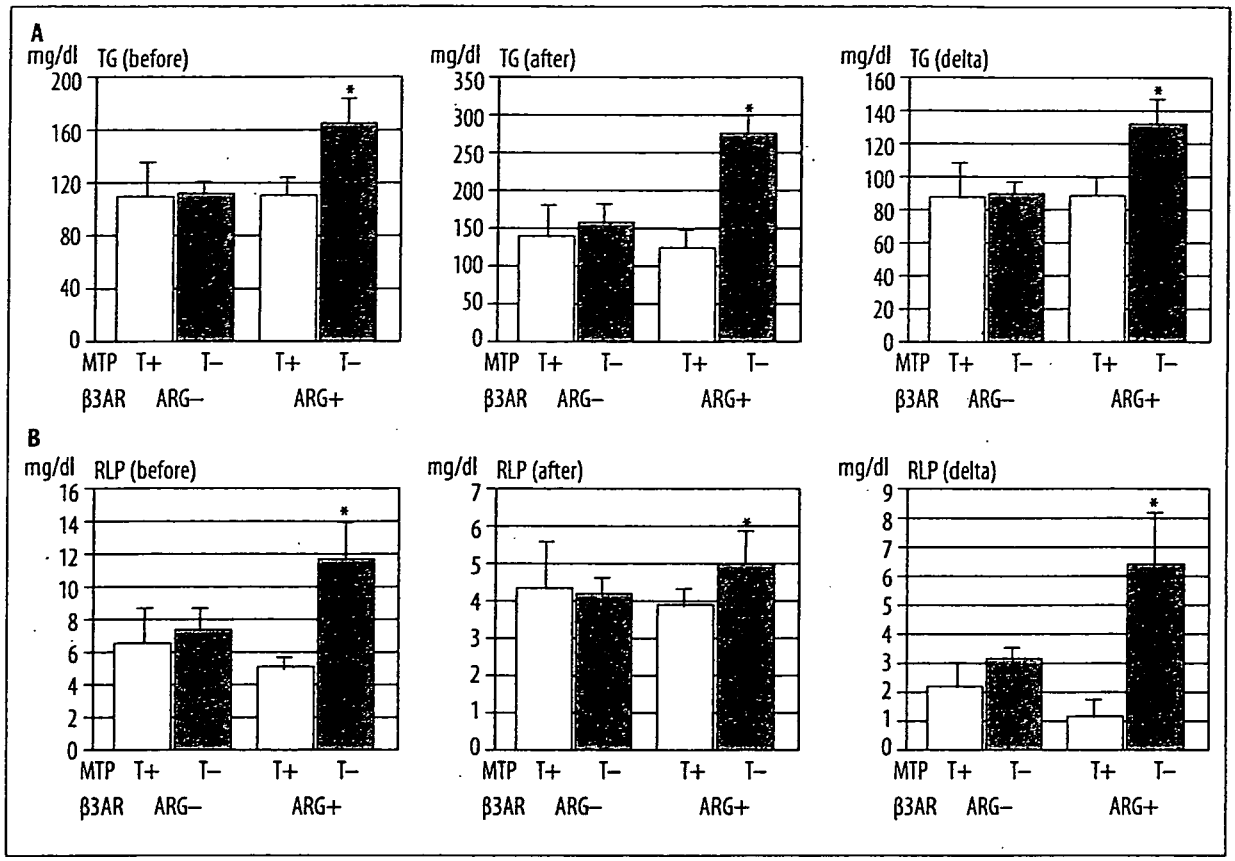

Figure 2. Plasma triglyceride levels during dietary loading tests in subjects classified according to MTP and β3AR polymorphisms. (**A**) Left panel, plasma triglyceride levels prior to dietary loading in each genotype group. Middle panel, plasma triglyceride levels after dietary loading in each genotype group. Right panel, differences in plasma triglyceride levels before and after dietary loading. (**B**) Left panel, plasma RLP levels prior to dietary loading in each genotype group. Middle panel, plasma RLP levels after dietary loading in each genotype group. Right panel, differences in plasma RLP levels before and after dietary loading. Blank columns indicates subjects with the T allele of MTP. White columns indicates subjects without the T allele. Arg+ indicates subjects with Arg allele of the Trp64Arg polymorphism in the β3AR gene. Arg- indicates subjects without the Arg allele. (*$P<0.05$).

a rat model of FCHL [16]. The syntenic region of one of these loci overlaps the human QTL for plasma concentration of small dense LDL. This locus includes many genes; however, we chose to focus on several genes involved in lipid metabolism, including the MTP gene. In the present study, we analyzed the effect of the MTP-493T variant and Trp64Arg polymorphism of the β_3AR gene in subjects undergoing dietary and glucose loading. MTP-493T variant was associated significantly with insulin resistance parameters such as, fasting plasma glucose, IRI and HOMA-R levels [29]. These findings are consistent with those of a previous study in French Canadian subjects [30]. Moreover, MTP-493T was associated with a lower postprandial serum triglyceride levels but not fasting plasma triglyceride levels. Determination of postprandial lipid level may unravel the influence of other genes on lipid metabolism.

In humans, β_3AR is expressed predominantly in adipose tissue and plays roles in regulation of resting metabolic rate and lipolysis [18,19]. In the present study, Arg allele was associated with fasting triglycerides, plasma glucose, insulin, and HOMA-R. These results are consistent with previously reported result [21] of reduced resting metabolic rate, hyperinsulinemia with obesity and reduced insulin sensitivity in subjects homozygous for the Arg allele.

We then analyzed lipid and glucose metabolism phenotypes with respect to MTP and β_3AR genotypes. Significant elevation of postprandial plasma triglyceride levels was obserbed only on Arrg+/T– subjects. β_3AR has been reported to be associated with insulin resistance and obesity [20,21,31], and St. Pierre et al. [29] reported the modulation of obesity and hyperinsulinemia on the MTP –493G/T polymorphism. Because β_3AR gene is known to associate with obesity and insulin resistance, we next analyzed the effect of BMI and HOMA-R on postprandial lipid levels of MTP variant. However, BMI and HOMA-R did not have similar effect as β_3AR genotype on postprandial plasma lipid levels.

The observed effect of the T–/Arg+ genotype on postprandial plasma triglyceride levels appears to be due to an interaction between the MTP and β_3AR genes. This interaction did not modify the effect of the β_3AR gene on obesity or insulin resistance.

We observed a significant increase in plasma glucose levels at 60 and 120 min after glucose loading in subjects with T–/Arg+ genotype, suggesting that an interaction between the genes contributes to glucose metabolism. Pihlajamaki et al. [32] reported the effect of the Arg genotype of the

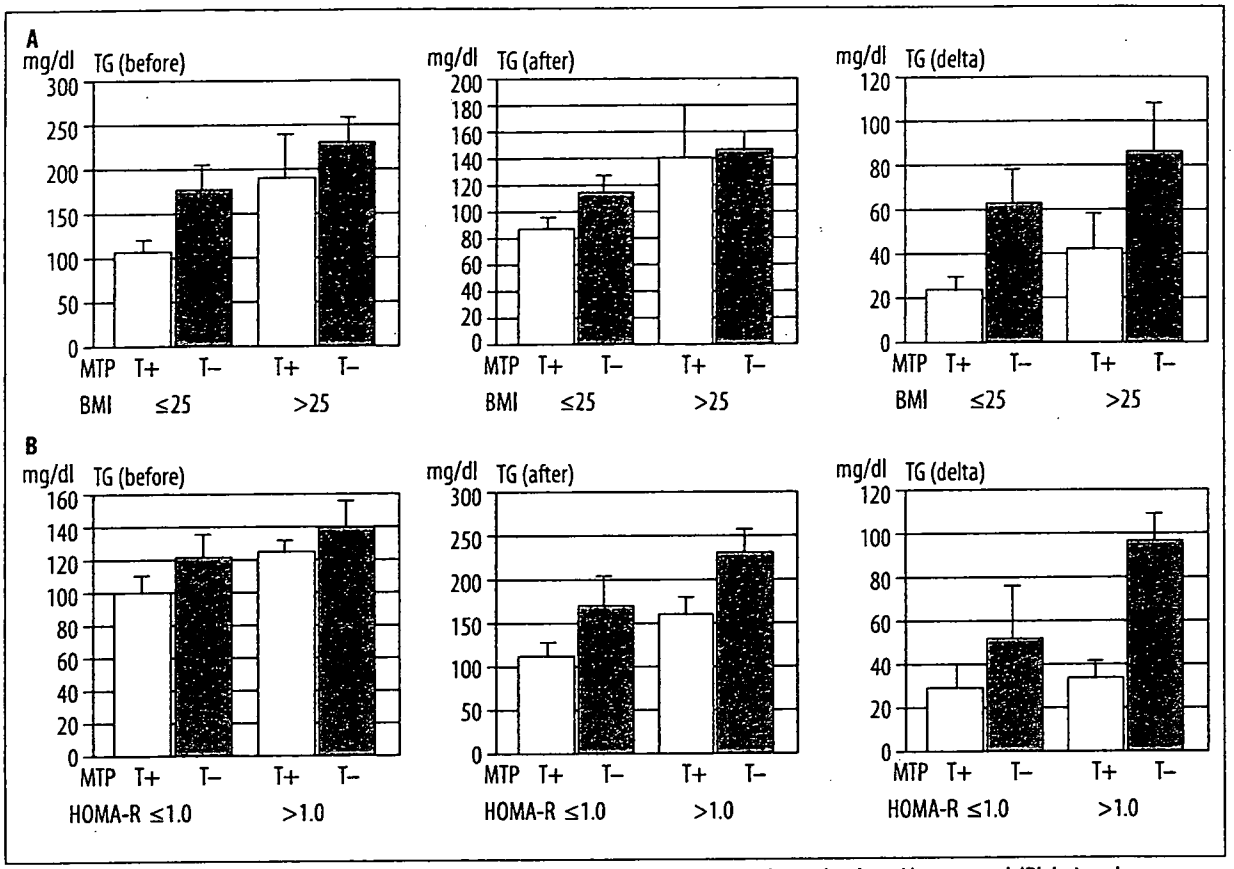

Figure 3. Plasma glucose and IRI levels during glucose tolerance test. Upper panel, plasma glucose levels and Lower panel, IRI during glucose tolerance test. Black solid line indicates T+/Arg+ subjects. Black dashed line indicates T+/Arg– subjects. Gray solid sine indicates T–/Arg+ subjects. Gray dashed line indicates T–/Arg– subjects. (*$p<0.05$).

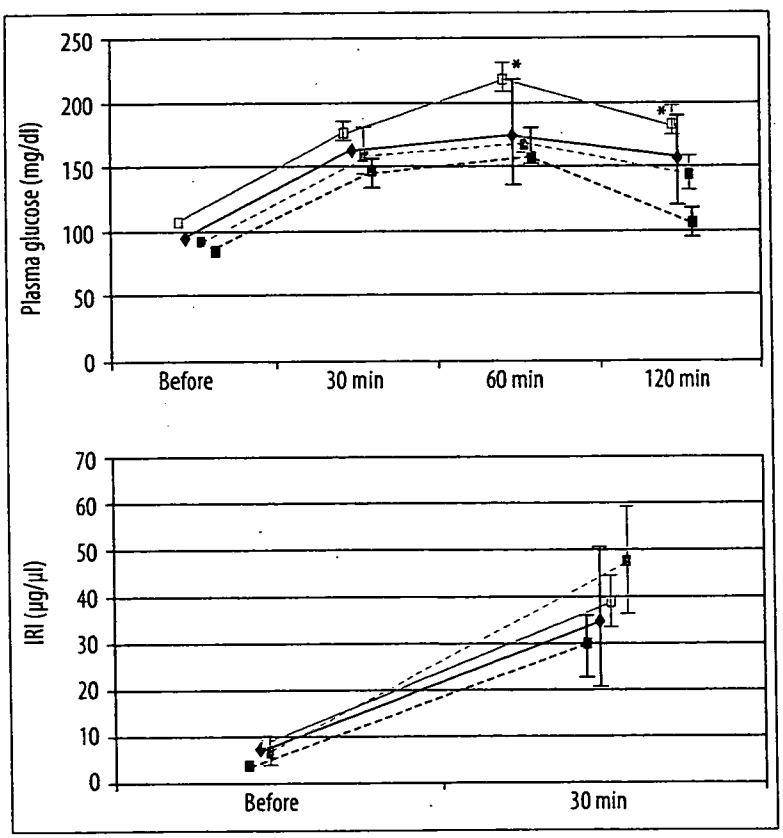

Figure 4. Plasma triglyceride levels in subjects classified by MTP genotype, BMI and HOMA-R during dietary loading test. (**A**) Left panel, plasma triglyceride levels prior to dietary loading in each genotype group. Middle panel, plasma triglyceride levels after dietary loading in each genotype group. Right panel, differences in plasma triglyceride levels before and after dietary loading. (**B**) Left panel, plasma RLP levels prior to dietary loading in each genotype group. Middle panel, plasma RLP levels after dietary loading in each genotype group. Right panel, differences in plasma RLP levels before and after dietary loading. Blank columns indicates subjects with the T allele of MTP. White columns indicates subjects without the T allele.

β_3AR gene on glucose oxidation rate and FFA levels in the fasting state plasma of patients with FCHL.

In the present study, we examined the differential effects of the MTP and β_3AR genotypes on fasting and postprandial triglyceride levels. Our results suggest that fasting and postprandial plasma triglyceride levels are under distinct genetic control. However, it is possible that analyzing postprandial plasma triglyceride levels may enhance and unravel the effect of genotypes on lipid metabolism. Studies of postprandial lipid levels appear to provide new information about lipid metabolism, and such information is important to our understanding of the mechanisms underlying metabolic syndrome and FCHL.

CONCLUSIONS

Both metabolic syndrome and FCHL are complex trait disease. The clinical phenotypes of these diseases are determined by complex interactions between several physiological phenomena resulting from genetic variations. In the present study, we identified one gene-gene interaction associated with human metabolic disorder. Further studies of genetic interactions in human diseases with complex phenotypes are necessary to clarify the mechanisms that underlie physiological phenotypes of such diseases.

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Original Article

Haplotype-Based Case-Control Study of the Association between the Guanylate Cyclase Activator 2B (GUCA2B, Uroguanylin) Gene and Essential Hypertension

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Uroguanylin (gene name: guanylate cyclase activator 2B, GUCA2B) is a peptide regulator of intestinal salt and water transport. It has been reported that the uroguanylin knockout mouse exhibits elevated blood pressure. Therefore, the GUCA2B gene is thought to be a susceptibility gene for essential hypertension (EH). Despite extensive studies, however, the relationship between the GUCA2B gene and EH has not yet been defined. The aim of this study was to assess the association between the human GUCA2B gene and EH. Using four single nucleotide polymorphisms (SNPs), we conducted a genetic association study in 281 EH patients and 279 age-matched normotensive (NT1) individuals. To derive more reliable data, we performed a duplicate case-control study in which we recruited another normotensive group (NT2). There was no significant difference in the overall distribution of alleles for any of the SNPs between the EH and NT1 groups, or between the EH and NT2 groups. Therefore, these four SNPs cannot be the genetic markers for EH. The occurrences of the C-A haplotype (rs883062-rs1047047) and the C-A-G haplotype (rs883062-rs1047047-rs2297566) were significantly higher in the EH group than in the NT1 group ($p<0.0001$) or the NT2 group ($p<0.0001$). These results suggest that the C-A haplotype and the C-A-G haplotype of the GUCA2B gene are the genetic markers for EH, and that GUCA2B or a neighboring gene might be a susceptibility gene for EH. (*Hypertens Res* 2007; 30: 789–796)

***Key Words*: haplotypes, guanylate cyclase activator 2B, single nucleotide polymorphism, association study, essential hypertension**

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This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (High-Tech Research Center, Nihon University), a research grant from the Alumni Association of Nihon University School of Medicine, and a grant from the Tanabe Biomedical Conference, Japan.

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Received September 11, 2006; Accepted in revised form April 19, 2007.

Introduction

While uroguanylin is expressed primarily in the mammalian intestine, it is also found in the kidney. It was identified because of its homology to the bacterial heat-stable enterotoxin (ST) and its ability to bind to the ST receptor, guanylate cyclase-C (GC-C) (*1*, *2*). ST is a small peptide that is produced by enterotoxigenic bacterial strains and that binds and activates the receptor GC-C. GC-C activation by ST during bacterial infection increases the intracellular levels of cGMP in enterocytes, causing a secretory diarrhea. GC-C is normally regulated by the endogenous peptide ligand, uroguanylin (*3*). Uroguanylin has local intestinal (paracrine) and endocrine functions, forming a potential enteric-renal link that is involved in coordinating salt ingestion *via* natriuresis (*4–6*). Previous studies on uroguanylin peptide suggest that it may function as an endocrine intestinal natriuretic hormone, as it has been found to circulate in the bloodstream (*7*, *8*). In addition, high-salt intake can cause an increase in uroguanylin mRNA (*9*, *10*), as well as an increase in the urinary excretion of uroguanylin (*11*). It has also been noted that there is an increase in uroguanylin levels in the circulation of patients with renal disease and congestive heart failure (*12*, *13*). Previous studies using mice that had a disruption of the genes responsible for the generation of uroguanylin have also provided information on the function of these genes. Blood pressure levels for mice with the targeted homozygous uroguanylin gene (−/−) were found to be significantly higher than those seen in the heterozygous (+/−) and wild types (+/+). These results suggest that uroguanylin is a very important factor in the physiological regulation of blood pressure (*1*).

The human GUCA2B gene that encodes uroguanylin is located on chromosome 1p33-p34 (*14*). The gene consists of three exons and two introns that are found within an overall length of 2.5 kb (*15*, *16*), and has several single nucleotide polymorphisms (SNPs). Although the GUCA2B gene is thought to be a susceptibility gene for hypertension, there have been no studies that have examined the association between the GUCA2B gene and essential hypertension (EH).

High blood pressure or hypertension affects 25% of most adult populations and is an important risk factor for death from stroke, myocardial infarction and congestive heart failure. Most hypertensive cases are classified as being primary and are referred to as EH. EH is thought to be a multifactorial disease (*17*).

The aim of this study was to assess the association between the human GUCA2B gene and EH using SNPs.

Methods

Subjects

This study included a group of 281 patients that were diagnosed with EH. A positive diagnosis required the patient to have a seated systolic blood pressure (SBP) above 160 mmHg and/or diastolic blood pressure (DBP) above 100 mmHg on three occasions within 2 months after their first medical examination. None of the patients were using antihypertensive medication and subjects diagnosed with secondary hypertension were excluded. We also included 279 normotensive (NT1) healthy individuals as controls. None of the NT participants had a family history of hypertension, and all had SBP and DBP below 130 and 85 mmHg, respectively. To derive more reliable data, we performed a duplicate case-control study in which we recruited another normotensive group (NT2). The NT2 subjects consisted of 285 essentially healthy elderly Japanese (mean age, 77.8±4.2 years). They were members of a group called the New Elder Citizen Movement in Japan, for which the physical and psychosocial characteristics have been previously described (*18*). The plasma high-density lipoprotein (HDL) cholesterol concentration and serum uric acid concentration data were not collected. A family history of hypertension was defined as a prior diagnosis of hypertension in grandparents, uncles, aunts, parents or siblings. Both groups were recruited from the northern area of Tokyo, and informed consent was obtained from each individual as per the protocol approved by the Human Studies Committee of Nihon University (*19*, *20*).

Biochemical Analysis

The methodology employed by the Clinical Laboratory Department of Nihon University Hospital was used to measure all plasma total cholesterol and HDL cholesterol concentrations, and serum creatinine and uric acid concentrations (*21*).

Genotyping

We selected four SNPs in the introns of the human GUCA2B gene as markers for the genetic association experiment (Fig. 1). The minor allele frequencies for each of the SNPs among the Japanese subjects were > 10%, which indicates that they all should be effective genetic markers. All SNPs were confirmed using the database for SNPs (dbSNP) on the NCBI website and the Applied Biosystems-Celera Discovery System. The accession numbers were as follows: rs883062 (C__1595618_10), rs1047047 (C__11291674_10), rs2297566 (C__1595598_10), and rs885846 (C__8909905_10) (Fig. 1). rs1047047 was located in exon 1 and exhibited no amino acid changes, thereby resulting in a silent mutation. rs883062 (C__1595618_10) was located upstream of exon 1. The other two SNPs were located in the intron.

Genotypes were determined using Assays-on-Demand kits (Applied Biosystems, Branchburg, USA) together with TaqMan® PCR. When allele-specific fluorogenic probes hybridize to the template during the polymerase chain reaction (PCR), the 5′ nuclease activity of the Taq polymerase can dis-

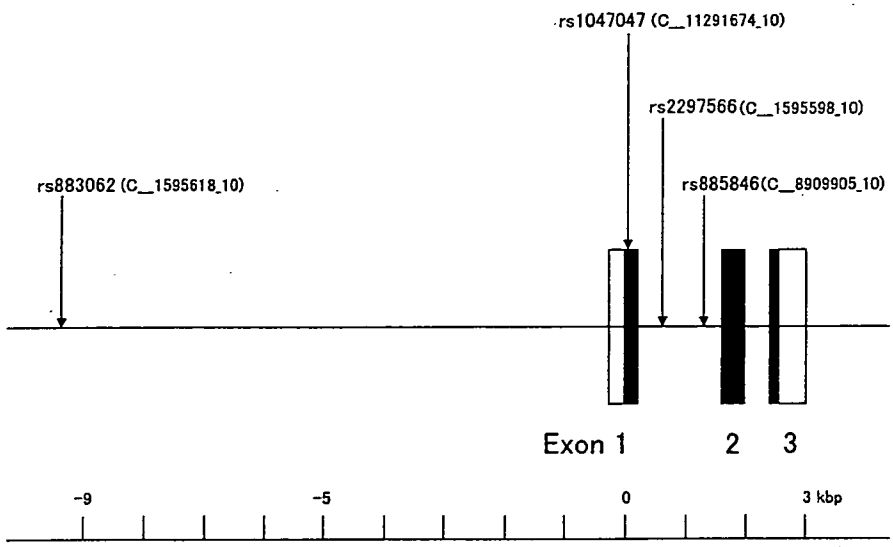

Fig. 1. *Organization of the human GUCA2B gene and location of the SNPs used for the association study. Closed black boxes indicate exons (coding region), closed white boxes indicate exons (non-coding region), and lines indicate introns.*

criminate alleles. Cleavage results in increased emission of a reporter dye that otherwise is quenched by the dye TAMRA. Each 5′ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe was labeled with a reporter dye (VIC and FAM) at the 5′ end and TAMRA at the 3′ end. Amplification by PCR was carried out using TaqMan Universal Master Mix (Applied Biosystems) in a 5 μL reaction volume with final total concentrations of 2 ng DNA, 900 nmol/L primer, and 200 nmol/L probe. Thermal cycling conditions consisted of 95°C for 10 min, and then 40 cycles of 92°C for 15 s and 60°C for 1 min in a GeneAmp 9700 system.

All 96-well plates contained 80 samples of unknown genotype, eight known allele 1 homozygotes, eight known allele 2 homozygotes, and eight reactions with reagents but no DNA. The homozygote and control samples without DNA were required for the SDS 7700 signal processing that is outlined in the TaqMan Allelic Discrimination Guide (Applied Biosystems). Direct sequencing, single-stand conformation polymorphism (SSCP), or denaturing high pressure liquid chromatography was used to confirm control sample genotypes. PCR plates were read on the ABI 7700 instrument using the SDS version 1.9 software package in the end-point analysis mode (Applied Biosystems). Genotypes were visually determined by comparison with the dye-component fluorescent emission data shown in the *X-Y* scatter-plot of the SDS software. Genotypes were also automatically determined by the signal processing algorithms in the software. The results of both scoring methods were saved to two output files for later comparison.

Haplotype-Based Case-Control Study and Linkage Disequilibrium Analysis

We performed a haplotype-based case-control study using the four SNPs. Based on the genotype data of the four genetic variations, the frequency of each haplotype was estimated using the expectation/maximization (EM) algorithm (*22*, *23*). In order to determine haplotype and linkage disequilibrium (LD), SNPAlyze version 3.2.3 was used (Dynacom Co., Ltd., Yokohama, Japan), which is available from the Dynacom website at http://www.dynacom.co.jp/products/package/snpalyze/index.html. Haplotypes with a frequency of <0.02 were excluded from the analysis.

Diplotype frequency was also estimated by SNPAlyze version 3.2.3. Clinical parameters were compared between the patients with and without one haplotype by using the diplotype data.

Statistical Analysis

Data are shown as the mean±SD. Differences between the EH and NT groups were assessed by analysis of variance (ANOVA) followed by a Fisher's protected least significant difference (PLSD) test.

Hardy-Weinberg equilibrium was assessed by a χ^2 analysis. When the sizes of the expected values were small (below 2.0), the genotypes were combined (*24*). The overall distribution of the SNP alleles was analyzed by 2×2 contingency tables, and the distribution of the SNP genotypes between the EH patients and NT controls was tested using a two-sided Fisher exact test and multiple logistic regression analysis. Statistical significance was established at $p<0.05$.