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V1A VASOPRESSIN RECEPTOR GENE SINGLE NUCLEOTIDE POLYMORPHISM AND ESSENTIAL HYPERTENSION, TYPE 2 DIABETES MELLITUS AND PLATELET AGGREGATION

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Abstract Candidate gene SNP study is a promising genetic approaches to complex common disorders. Arginine vasopressin (AVP), a peptide hormone released from the posterior pituitary, has been suggested to play important roles in the regulation of blood pressure, glycogenolysis and platelet aggregation through G protein coupled V1a receptor (V1aR). Thus, polymorphisms in the V1aR gene have been prospective as possible genetic markers for essential hypertension, type 2 diabetes mellitus and divergent platelet aggregation response to AVP. We identified 4 novel single nucleotide polymorphisms (SNPs) in the promoter region of the V1aR gene and named according to the upstream locations such as, -6951G/A, -4112A/T, -3860T/C, and -242C/T. We investigated the association of 4 SNPs of the V1aR gene in 365 hypertensive and 255 healthy subjects, 186 T2DM patients and 188 non-diabetic control subjects (CS), and 33 young healthy volunteers living in the Aomori prefecture. Significant association was identified between SNP at -6951 and hypertension in nonobese individuals, at -6951 and type 2 diabetes mellitus. Positive association was also identified between nonobese hypertension and haplotype H3. Significant association was demonstrated between SNP at -6951 and glycemic status in young healthy subjects. However, there was no significant association in the AVP-induced platelet aggregation with V1aR gene variants. The study suggests V1aR gene variants as increased risk for hypertension in nonobese and type 2 diabetes mellitus in the Aomori population; however, might not be useful as genetic markers for platelet aggregation heterogeneity.

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Key words: vasopressin V1a receptor; SNPs; essential hypertension; type 2 diabetes mellitus; platelets aggregation heterogeneity.

Introduction

Hypertension and type 2 diabetes mellitus are major cardiovascular risk factors with genetic and environmental components. Platelet aggregation also plays a central role in the pathogenesis of acute thrombosis in coronary heart disease.[1-6] Extensive studies have been carried out to search candidate genes responsible for susceptibility to essential hypertension, type 2 diabetes mellitus and to clarify the role of genes in platelet aggregation. However, genes involved in the pathogenesis of these disorders remain to be identified.

Arginine vasopressin (AVP), the antidiuretic hormone released from the posterior pituitary, is involved in diverse physiological actions, including contraction of vascular smooth

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muscle cells and mesangial cells, stimulation of hepatic glycogenolysis, antidiuresis in the kidney, aggregation of platelets and modulation of adrenocorticotropin (ACTH) release from the pituitary.[7] AVP mediates its physiological effects through G protein-coupled receptors. The human AVP receptors have been cloned and found to be differentially expressed: the V1a in vascular smooth muscle, liver, blood platelets, brain, adrenal cortex, and renal mesengial cells, the V2 in kidney and the V1b in pituitary.[8-11]

AVP exerts its vasoconstrictor action through V1a receptor (V1aR) on vascular smooth muscles,[12,13] to increase vascular tone and modulate baroreceptor sensitivity by central nervous system V1aR.[14] V1aR plays an important role in the blood glucose regulation. In the liver, it promotes glycogenolysis and gluconeogenesis. Hypothalamic AVP facilitate hyperglycemic responses initiated by peripheral signals through the hepatic activation of V1aR.[15-22] Human platelet aggregation response of to AVP shows heterogeneity,[23] which is thought to be under the genetic influence. AVP has also been reported to stimulate platelet aggregation by platelet V1aR. All of these physiological functions allowed V1aR gene a reasonable candidate for the study of its involvement in human essential hypertension, type 2 diabetes mellitus as well as clarifying heterogeneity of the aggregation response of human platelets to AVP.

The V1aR gene is located on chromosome 12 and maps to region 12q14-15. It consists of two coding exons, exon 1 with 2 kb of 5'-untranslated region and exon2 with 1 kb of 3'-untranslated region, separated by a 2.2-kb intron. A 8.9-kb ApaI fragment correspondes to the 5′ flanking promoter DNA and the first 32 nucleotides of the V1aR gene.[24]

There are several reports on microsatellite motifs and novel single nucleotide polymorphisms (SNPs) in the V1aR gene and its 5′ up stream region. Thibonnier et al[25] found several DNA microsatellite motifs in French Caucasian. One is located within the intron. Three other microsatellites are present in the 5′ flanking DNA of the V1aR gene. The study explored whether human V1aR gene might be involved in human essential hypertension. However, the study revealed no association with essential hypertension.[25] Study for searching variations in eight human gene encoding G protein-coupled receptors (GPCRs) in the Japanese population detected 20 variations in the V1aR gene. Among those, some were reported in other studies, the rests were novel. Nineteen genetic variations are in the 5′ flanking region, 5′ untranslated region of exon 1, intron, 3′ untrnaslated region of exon 2 and 3′ flanking regions. Only one is found in exon 1; however, the change of C to T caused no amino acid change.[26] Ten SNPs in the V1aR gene have also been reported. Three microsatellites in the V1aR gene, two in the promoter region (RS1 and RS3) and an intronic microsatellite, were found to be associated with autism spectrum disorders.[27-29] We also investigated genetic variations in the V1aR gene in native Japanese general residents living in the northern most rural district of Japan main island, Aomori prefecture. We identified 4 novel SNPs in the promoter region of V1aR gene and named according to the upstream locations such as -6951G/A, -4112A/T, -3860T/C and -242C/T.

In the present study, we investigated whether these 4 SNPs of V1aR gene could be associated to confer susceptibility to essential hypertension, type 2 diabetes mellitus as well as platelet aggregation heterogeneity to AVP. We also looked at the effect of SNPs on glycemic status.

Subjects and Methods

The study of V1aR SNPs with essential hypertension was comprised of total 620 subjects including 365 hypertensive (HT) and 255 healthy subjects (HS). Hypertension was defined as an average systolic blood pressure (SBP) of

140 mmHg or higher, an average diastolic blood pressure (DBP) of 90 mmHg or higher, and/or current use of antihypertensive medications. SBP and DBP of all healthy subjects were less than 140 and 90 mmHg, respectively. Healthy subjects had no history of hypertension, body mass index (BMI) of <25 kg/m^2 and were without any medical treatments. Additional selection criteria for both hypertensive and healthy subjects were the absence of: (1) diabetes mellitus (fasting blood glucose >6 mmol/L or use of insulin or oral hypoglycemic agents), (2) renal dysfunction (serum creatinine >180 mmol/L), (3) liver disease, (4) malignancy, (5) pregnancy, and (6) substance abuse, including alcohol.

The study of V1aR SNPs with type 2 diabetes mellitus was comprised of a group of 374 subjects, 186 patients with type 2 diabetes mellitus (T2DM) and 188 age- and sex-matched non-diabetic control subjects (CS). The study included another group of 33 young healthy subjects [Age: 22.0±1.0 yrs]. The diagnosis of diabetes mellitus was set according to the criteria of the Japanese Diabetes Association. Namely, patients with diabetes mellitus had fasting plasma glucose concentration of ≥126 mg/dl repeatedly. Otherwise, plasma glucose concentration of 2 hours after oral 75 g glucose load was ≥200 mg/dl. The diagnosis of T2DM was based on the 1998 WHO criteria.[30] Only patients with treatment on diet or exercise and oral hypoglycemic agent were recruited. Control subjects had no history of diabetes and hypertension and were without any medical treatments.

The study for the association with the platelet responsiveness to AVP comprised 33 young healthy volunteers [age (mean±SD):22±1]. All subjects were without of diabetes mellitus, hypertension, hypercholesterolemia, smoking history and hyperlipidemia. None had any history of atherosclerotic disease, abnormal bleeding, and arterial or venous thrombotic disorders. All subjects had no history of ingesting ethanol, aspirin, or other medications.

All subjects in the different studies gave informed consent. The ethics committee of the institute approved the research protocols.

Genotype screening were performed on peripheral blood extracted genomic DNA using PCR restriction fragment length polymorphism (PCR-RFLP) or PCR allele specific amplification (PCR-ASA). Primers sequences and locations were according to Hasan et al.[31] The restriction enzymes used for RFLP were Bfa I for -6951 G/A, Tsp509 I for -3860 T/C and Pst I for -242 C/T, respectively. For -4112 A/T, PCR was performed using forward primer allele 1 (A), forward primer allele 2 (T) and common reverse primer. The allele specific amplified product was identified by the electrophoresis migration.

Data are expressed as means±SD unless otherwise stated. Clinical characteristics between groups were analyzed by Student's t-test and one-way analysis of variance (ANOVA). χ^2 analysis and Fisher's exact test was applied to examine the differences in genotypes, alleles, haplotypes, as well as haplotype combinations between the groups. Logistic regression analysis was performed to asses the independent contribution of confounding factors. Pearson's correlation analysis evaluated the relationship between AVP and ADP in inducing platelet aggregation. Haplotyps, Hardy-Weinberg equilibrium and linkage disequilibrium co-efficient (D′) were estimated by using genetic analysis software (Helix Tree, version 4.0.3) [www.goldenhelix.com/em_algorithm.html]. P value of less than 0.05 was considered statistically significant.

Results and Discussion

Mean SBP and DBP at the time of blood collection were 120.2±11.3/72.5±8.8 mmHg in HS, and 150.9±20.0/89.2±12.2 mmHg in HT, respectively. Thus, SBP and DBP were significantly higher in HT. The baseline

characteristics of age and sex were not significantly different between the two groups. However, BMI, serum total cholesterol and serum total HDL cholesterol were significantly different between HT and HS.

Genotype distributions at each of 4 polymorphic sites of V1aR gene were in the Hardy-Weinberg equilibrium in all subjects. The overall distribution of genotypes and alleles of 4 SNPs did not differ significantly between HS and HT (data not shown). However, when hypertensives were stratified according to the obesity (BMI≥25.0 kg/m^2)[32], there was significant association between genotypes and alleles at -6951 and hypertension. Among hypertensives, there were 201 subjects with BMI<25.0 kg/m^2 and regarded as nonobese hypertensive (NOB-HT) (mean age, 62.0±9.8 years; male/female, 82/119; mean BMI, 22.4±1.8 kg/m^2); while there were 164 subjects with BMI≥25.0 kg/m^2 and regarded as obese hypertensive (OB-HT) (mean age, 59.1±9.2 years; male/female, 61/103; BMI, 27.8±2.4 kg/m^2). The prevalence of GA+AA genotype of SNP -6951 was significantly higher in NOB-HT compared with HS (P=0.016). The frequency of minor A allele of SNP -6951 was also significantly higher in NOB-HT compared with HS (P=0.019) (Table 1).

Except for the significantly higher prevalence of C allele at -3860 (T allele vs. C allele: P=0.037), genotype and allele distributions of 3 other SNPs of V1aR did not show any significant differences between the two groups (data not shown). Genotype and allele distributions of 4 SNPs of V1aR did not show any significant differences between healthy and OB-HT subjects (data not shown). In all subjects, these 4 SNPs were in strong linkage disequilibrium with each other. Therefore, we determined haplotypes based on 4 SNPs of V1aR. The distributions of major haplotypes (H1-H5) between the HS and NOB-HT group revealed significantly higher frequency of H3 haplotype in NOB-HT (P=0.048) that include the -6951 variant (Table 1). The significant association (P=0.028) of SNP -6951 G/A with hypertension in nonobese remained after adjustment for confounding factors (Table 2).

These results suggest that V1aR gene variation at -6951 confer susceptibility to hypertension in nonobese individuals. Case-control and linkage study of V1aR gene microsatellite polymorphisms in French Caucasian showed no association with human essential hypertension[25]. We also found no association between V1aR gene SNPs and hypertension as a whole. However, when cases were subdivided into nonobese and obese hypertensives, an association was observed between V1aR SNP and hypertension in a subsample of nonobese individuals.

The factor that led us to stratify hypertensives is genetic heterogeneity. Genetic heterogeneity may arise due to disease etiology and ethnicity. Essential hypertension is heterogeneous disorder and several underlying mechanisms may contribute to develop it. Obesity is one of those. Therefore, categorizing hypertensives according to the clinically defined subtypes would reduce the genetic heterogeneity and improve the power of the study. It has become evident that obesity-associated hypertension may be genetically distinct from hypertension in nonobese individuals. Pausova et al[33] performed genome-wide scans in French Canadians and found that the loci for the obesity-associated hypertension were significantly different from those for nonobese hypertension. The HYPERGENE studies already implicated the association of angiotensinogen gene in the development of hypertension in individuals with BMI<27 kg/m^2[2, 34,35]. Hepatocyte growth factor gene polymorphism was reported to confer susceptibility to essential hypertension in lean subjects[36]. Dissecting hypertensives by ethnicity has been also shown to reduce the level of genetic heterogeneity. Morris[37] stated that Pausova et al[33] succeeded to improve the degree of genetic

Table 1 Comparison of genotype, allele frequencies of SNP -6951G/A and haplotype (combined alleles of -6951 G/A, -4112 A/T, -3860T/C, and -242 C/T) frequencies of V1aR gene between healthy subjects (HS) and nonobese hypertensives (NOB-HT)

SNP	Genotypes/ Alleles	HS (n = 255)	NOB-HT (n = 201)	P	OR (95% CI)
−6951 G/A					
	GG	182 (71.0)	121 (60.0)		
	GA+AA	73 (29.0)	80 (40.0)	**0.016**	1.65 (1.11−2.44)
	G	428 (84.0)	312 (78.0)		
	A	82 (16.0)	90 (22.0)	**0.019**	1.51 (1.10−2.10)
Haplotypes					
H1	g-a-t-c	231 (0.453)	168 (0.418)	0.32	1.15 (0.89-1.50)
H2	g-a-c-c	114 (0.224)	94 (0.234)	0.77	0.94 (0.69-1.29)
H3	a-a-c-c	82 (0.161)	86 (0.214)	**0.048**	0.70 (0.50-0.98)
H4	g-t-t-t	65 (0.127)	43 (0.107)	0.40	1.22 (0.81-1.83)
H5	g-t-t-c	17 (0.033)	6 (0.015)	0.12	2.28 (0.89-5.82)

Values in case of genotype and allele frequencies are actual counts (%). Values are counts (fraction) in case of haplotype frequencies. Haplotypes are shown as combined alleles of -6951 G/A, -4112 A/T, -3860T/C, and -242 C/T. The results of haplotypes with very rare frequencies (≤ 1.0%) in HS and NOB-HT were not shown. V1aR, vasopressin V1a receptor; SNP, single nucleotide polymorphism; NOB-HT, nonobese hypertensive; OR, odds ratio; CI, confidence interval.

Table 2 Effect of confounding factors on the prevalence of hypertension in non obese by multiple logistic regression analysis

Variables	df	Wald χ^2	P	OR	95% CI
Age	1	7.4	0.007	1.03	1.01 − 1.05
Sex	1	3.7	0.055	0.66	0.44 − 1.01
BMI	1	13.4	< 0.001	1.22	1.1 − 1.35
Total cholesterol	1	1.0	0.313	1.0	1.0 − 1.01
HDL-cholesterol	1	0.001	0.974	1.0	0.99 − 1.01
−6951 G/A[a]	1	4.8	**0.028**	1.57	1.05 − 2.35

df, degree of freedom; BMI, body mass index; HDL, high-density lipoprotein; -6951 G/A, single nucleotide polymorphism at -6951 position of vasopressin V1a receptor gene. OR, odds ratio; CI, confidence interval. [a]The relative risk associated with carriers of GA+AA of -6951 G/A has been estimated vs. GG genotype.

homogeneity by recruiting isolated population from the geographically remote French-Canadian Saguenay/Lac-St-Jean region of Quebec. The results by Pausova et al[33] are contrasts with the 2 largest genome scans for hypertension, the National Heart, Lung and Blood Institute Family Blood Pressure Program (NHLBI-FBP)[38] and the British Investigation of the Genetics of Hypertension (BRIGHT)[39] studies, which failed to find even a single locus that attained genome-wide significance. In the present study, we also recruited native Japanese residents who were living in traditional, conservative, and agriculture based life style in the rural district, Aomori prefecture. Their ancestors lived there over the past several centuries. Therefore, it is likely that our relatively small, isolated population has been also spared the level of genetic "noise" present in mixed populations elsewhere.

There was a significant difference (P=0.048) in -6951G/A genotype distribution between T2DM and CS groups (Table 3).

Although A allele of −6951 SNP was found to be more frequent in T2DM, this was not

Table 3 Comparison of genotype and allele frequencies of SNP -6951G/A of V1aR gene between control subjects (CS) and patients with type 2 diabetes mellitus (T2DM) and effect of confounding factors on the prevalence of type 2 diabetes mellitus by multiple logistic regression analysis

SNPs	Genotypes/ Alleles	CS (n = 188)	T2DM (n = 186)	P	OR (95% CI)
−6951G/A					
	GG	140 (74.0)	120 (65.0)		
	GA+AA	48 (26.0)	66 (35.0)	**0.048**	1.60 (1.03 − 2.5)
	G	321 (85.0)	299 (80.0)		
	A	55 (15.0)	73 (20.0)	0.09	1.42 (0.97 − 2.1)
Logistic regression					
Variables	df	Wald χ^2	P	OR (95% CI)	
Age	1	1.22	0.27	1.01 (0.99 − 1.03)	
Sex	1	0.56	0.46	0.85 (0.57 − 1.29)	
−6951 G/A[a]	1	4.15	**0.04**	1.59 (1.02 − 2.49)	

Values in case of genotype and allele frequencies are actual counts (%). V1aR, vasopressin V1a receptor; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; df, degree of freedom. [a]The relative risk associated with carriers of GA+AA of -6951 G/A has been estimated vs. GG genotype.

significant (Table 3). Logistic regression also confirmed the independent significant association of the SNP −6951G/A with type 2 diabetes mellitus (P=0.04) after adjustment for age and sex. Genotype and allele distributions of 3 other SNPs of V1aR did not show any significant differences between the T2DM and CS groups (data not shown). The distributions of major haplotypes (H1-H5) between the two groups showed no significant differences between CS and T2DM. Analysis of haplotype combinations showed that H2/H3 heterozygote was more frequent in T2DM than in CS (8.6% versus 3.7%). H1/H2 heterozygote appeared more frequently in CS than in T2DM (22.0% versus 14.5%). SNP -6951G/A demonstrated a significant relation with the glycemic status in young healthy subjects. GA+AA carriers of SNP -6951G/A had significantly (P < 0.001) higher HbA_{1C} (4.66±0.13) compared to GG carriers (4.26±0.24) (Figure 1A).

However, HbA_{1C} did not show any significant differences for each of 3 other SNPs of V1aR. In young healthy subjects, HbA_{1C} levels in carriers having haplotype combinations of H1/H1 and H1/H2 (more frequent in control subjects) were 4.25±0.171 and 4.11±0.273, significantly lower (P<0.01 and P<0.001) from that in all other combinations (4.53±0.23) (Figure 1B).

These results suggest the importance of -6951G/A SNP as genetic marker of type 2 diabetes mellitus and haplotypes in predicting type 2 diabetes mellitus. There are substantial evidences to suggest that AVP plays a crucial role in the regulation of blood glucose through V1aR[18-22,40]. However, the present study seems to be the first genetic association study between V1aR gene polymorphisms and type 2 diabetes mellitus.

The maximum aggregation responses to AVP varied from 0% to 97% with a mean value of 47.88±36.85%, whereas those to ADP varied from 52% to 98% with mean value of 79.82±12.67%. There was a significant correlation (r=0.59; P<0.001) between responses to AVP and those to ADP. However, there was no significant association in the AVP-induced aggregation between the subjects with and without variant alleles of each 4 SNPs (data not shown). These results suggest that our identified V1aR gene

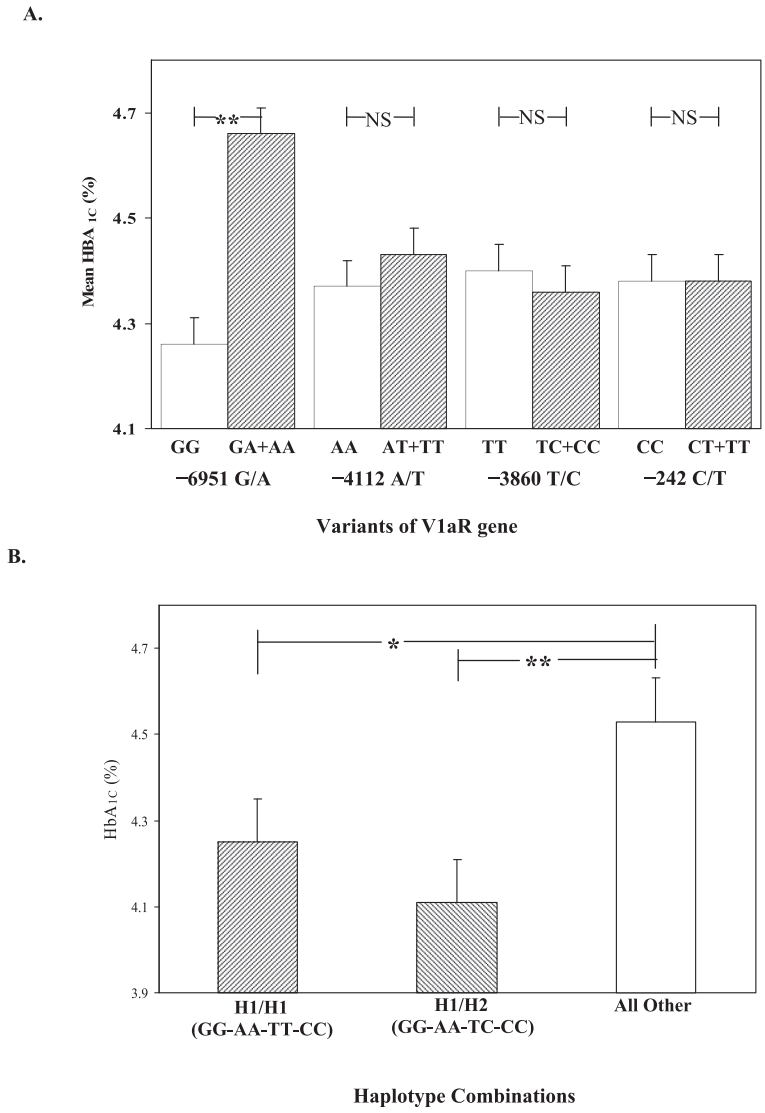

Figure 1 Effect of 4 SNPs of V1aR gene on glycemic status. A. HbA_{1C} Status and V1aR SNPs in Young Healthy Subjects. B. HbA_{1C} Status and haplotype combinations based on 4 SNPs of V1aR gene in Young Healthy Subjects. P<0.01; ** P<0.001

variants may not be useful as genetic markers for platelet aggregation heterogeneity. This study was the first to investigate the association of V1aR SNPs with the heterogeneity of platelet aggregation in response to AVP. We confirmed that platelet aggregation response to AVP varies among individuals. Significant correlation between platelet aggregation response to AVP and those to ADP suggests that they may share a common pathway in inducing platelet aggregation. Activation of phospholipase C via Gq is indispensable for both AVP and ADP-induced platelet aggregation[9,41,42].

In conclusion, the present study demonstrates a significant association of the -6951G/A SNP with nonobese hypertension and type 2 diabetes mellitus. Four SNPs of V1aR might not be useful as genetic marker for platelet aggregation heterogeneity. Future studies will be needed to investigate the functional relevance of V1aR gene polymorphisms in these disorders.

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