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ORIGINAL ARTICLE

ROLE OF VASOPRESSIN V1a RECEPTOR IN HYPERTENSION, DIABETES MELLITUS AND PLATELET FUNCTION: GENETIC POLYMORPHISM STUDY

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Abstract Arginine vasopressin (AVP), a peptide hormone released from the posterior pituitary, has been suggested to play important roles in cardiovascular regulation, glycogenolysis and platelet aggregation through its V1a receptor (V1aR). In this study, we investigated the association of 4 novel single nucleotide polymorphisms (SNPs) in V1aR gene (−6951G/A, −4112A/T, −3860T/C and −242C/T) with hypertension, diabetes mellitus (DM) and glycemic status, and platelet responsiveness to AVP. Genotypes were determined in 365 hypertensives and 255 healthy subjects, 186 patients with type 2 DM (T2DM) and 188 non-diabetic control subjects (CS), and 33 young healthy subjects in the Aomori prefecture. A significant association was found between the SNP −6951G/A and hypertension in nonobese individuals. Multiple logistic analysis demonstrated SNP −6951G/A as an independent risk factor for nonobese hypertension. One haplotype H3 (a-a-c-c) was characteristic to hypertension in nonobese. A significant association was found between the SNP −6951G/A and type 2 DM. Logistic regression also confirmed the significant association of the SNP −6951G/A with type 2 DM. There were a couple of characteristic distributions of heterozygous haplotype combinations in T2DM. In young healthy subjects, GA+AA carriers of SNP −6951 showed significantly higher HbA_{1C} levels than in GG. Platelet aggregation responses to AVP were identical between wild types and variants of any SNPs. These results suggest that −6951G/A SNP and haplotypes created by 4 SNPs of V1aR gene might confer susceptibility to essential hypertension in nonobese individuals and type 2 DM. However, these SNPs might not be useful as genetic marker for platelet aggregation heterogeneity.

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Key words: vasopressinV1a receptor; SNPs; essential hypertension; nonobese Japanese; type 2 diabetes mellitus; platelet aggregation heterogeneity.

原　著

高血圧，糖尿病，血小板機能におけるバゾプレシンV1a受容体の役割

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抄録　アルギニンバゾプレシン（AVP）は下垂体から分泌されるペプチドホルモンで，V1a受容体（V1aR）を介して心血管系の制御，グリコーゲン分解，血小板凝集に重要な役割を果たしている．本研究では，V1aRの新たな４個の一塩基多型（−6951G/A，−4112A/T，−3860T/C，−242C/T）と高血圧症，２型糖尿病と血糖レベル，AVPに対する血小板凝集反応との関連を検討した．対象は，青森県内の高血圧症365例と健常対照者255例，２型糖尿病186例と非糖尿病対照者188例，健常若年成人33例である．層別化解析により4多型のうち−6951G/A多型と非肥満者における高血圧との連関が認められた．多変量解析では−6951G/A多型は非肥満者高血圧の独立した危険因子であることが示唆され，高血圧との関連の強いハプロタイプが存在した．２型糖尿病に対しても−6951G/A多型は多変量解析上独立した危険因子と認められ，また，関連の強い２種のハプロタイプ結合が存在した．さらに，健常若年成人における−6951G/A多型の変異型では，野生型のホモよりヘモグロビンA1cが高値であった．一方，AVPによる血小板凝集反応の個体差と４個の一塩基多型とのあいだには関連性が認められなかった．以上の結果から，V1aR−6951G/A多型の変異型では，非肥満者高血圧および２型糖尿病の疾患感受性が高い可能性が示唆された．しかし，検討したこれらのV1a受容体遺伝子多型は血小板凝集反応性の遺伝子マーカーにはなりにくいと考えられた．

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Introduction

Both hypertension and diabetes mellitus (DM) have been shown to increase the risk of cardiovascular morbidity and mortality. Platelet aggregation also plays a central role in the pathogenesis of acute thrombosis in coronary heart disease[1-4].

Essential hypertension and type 2 DM are heterogeneous polygenic disorders that interact with multiple environmental factors[5,6]. Platelet aggregation heterogeneity among individuals in response to physiological agonist is also thought to be under the genetic influence[7,8]. Extensive efforts have been made to search candidate genes responsible for the development of essential hypertension and type 2 DM and to clarify the role of genes in platelet aggregation. However, role of genes in the development of these disorders are yet to be determined.

Arginine vasopressin (AVP), the antidiuretic hormone secreted from the posterior pituitary, plays an important role in cardiovascular regulation through multiple actions such as vasoconstriction, baroreceptor modulation, and antidiuresis[9-11]. Role of AVP in the maintenance of blood pressure is well documented in several conditions including upright posture, dehydration, hemorrhage, adrenal insufficiency, cardiac failure as well as surgical damage[11-13]. The significant role of AVP has been documented in several models of genetic and experimental hypertension, such as spontaneously hypertensive rat, the DOCA-salt hypertensive rat and the deoxymethasone hypertensive rat[11,14,15]. AVP has been implicated to mediate glycogenolysis in the liver[16,17]. AVP has also been reported to stimulate platelet aggregation. The aggregation response of human platelets to AVP shows heterogeneity[18].

AVP mediates its physiological effects through G protein-coupled receptors. 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 the pituitary[19-22]. AVP exerts its vasoconstrictor action through V1a receptor (V1aR) on vascular smooth muscles[19,20], to increase vascular tone and modulate baroreceptor sensitivity by central nervous system V1aR[11]. V1aR has been implicated to mediate glycogenolysis in liver[16,17]. In addition, hypothalamic AVP acting on V1aR in the nucleus of the tractus solitarius (NTS) facilitate hyperglycemic responses initiated by peripheral signals and it is suggested that hepatic activation of V1aR is essential for this hyperglycemic response[23,24]. V1aR, upon stimulation induce platelet aggregation. Therefore, all of these physiological functions allowed V1aR gene a reasonable candidate for the study of its involvement in human essential hypertension, type 2 DM as well as clarifying heterogeneity of the aggregation response of human platelets.

The V1aR gene is located on chromosome 12 and maps to region 12q14-15. The V1aR comprises 418 amino acids of the open reading frame and its gene is included entirely within a 6.4-kb EcoRI fragment with two coding exons separated by a 2.2-kb intron. The first exon also contains 2 kb of 5′-untranslated region, and the second exon includes 1 kb of 3′-untranslated region. Human chromosome 12-specific genomic library screening led to the isolation of an additional 8.9-kb ApaI fragment corresponding to the 5′ flanking promoter DNA and the first 32 nucleotides of the V1aR gene[25].

There are several reports on microsatellite motifs and novel single nucleotide

polymorphisms (SNPs) in the V1aR gene and its 5′ upstream region[26-29]. 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. However, it is yet uncertain whether V1aR gene variants represent marker of human essential hypertension, type 2 DM and platelet responsiveness to AVP.

Therefore, the present study was ascertained to explore whether the identified 4 SNPs of V1aR gene could be associated to confer susceptibility to essential hypertension, type 2 DM as well as platelet aggregation heterogeneity to AVP. The study also looked at the effect of SNPs on glycemic status.

Methods

Study Subjects

All subjects were recruited from native Japanese residents living in the northern most rural district of the Japan main island Honsu, Aomori prefecture. All samples were taken and clinical examinations performed in accordance with the Helsinki declaration. All subjects in the different studies gave informed consent. The ethics committee of the institute approved the research protocols.

The study of V1aR SNPs with essential hypertension was comprised of total 620 subjects including 365 hypertensives (HT) and 255 healthy subjects (HS). All subjects visited the community based rural health checkup. The study subjects underwent a physical examination that included height, weight, body mass index and blood pressure. Inclusion was based on both clinical characteristics and routine laboratory tests performed at the Department of Laboratory Medicine of Hirosaki University Hospital. Blood pressure was measured in sitting position after at least 10 minutes of rest using a mercury sphygmomanometer. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were the means of three measurements by well-trained doctors (recorded > 3 minutes apart). 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. HT diagnosed as secondary hypertension by routine examinations, were excluded. SBP and DBP of all HS were less than 140 and 90 mmHg, respectively. HS 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 HT and HS 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 definition of obesity was set to BMI of 25 kg/m^2 or higher according to the Japan Society for the Study of Obesity (JASSO)[30].

The study of V1aR SNPs with type 2 DM consisted of 374 subjects, 186 patients with type 2 DM (T2DM) [age (mean ± SD): 62.8 ± 10.7 yrs; sex (male/female): 99/87] and 188 age- and sex-matched non-diabetic control subjects (CS) [age (mean ± SD): 61.4 ± 10.8 yrs; sex (male/female): 95/93]. The study included another group of 33 young healthy subjects [age (mean ± SD): 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 type 2 DM was based on the 1998 WHO criteria[31]. 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 subjects [age (mean ± SD): 22 ± 1.0 yrs]. All subjects were free of diabetes mellitus, hypertension, hypercholesterolemia, smoking history and hyperlipidemia. None had any history of atherosclerotic disease, abnormal bleeding, and arterial or venous thrombotic disorders. The subjects were asked to fast overnight and to refrain from intensive exercise. All subjects had no history of ingesting ethanol, aspirin, or other medications known to alter platelet function for at least one week.

Biochemical Analysis

Fasting peripheral blood was collected and serum was separated within 2 hours and stored at −70℃. Total cholesterol and HDL-cholesterol were measured on automated analyzer TBA-200FR (Toshiba, Tokyo, Japan) by enzymatic methods. HbA_{1C} was determined by high-pressure liquid chromatography (HLC-723GHbV A1C2.2 Automated Glycohemoglobin Analyzer; TOSOH Bioscience Inc., San Francisco, CA, USA).

Platelet Aggregation Tests

To avoid platelet activation in vivo due to physical exercise, the study subjects were at rest for at least 15-20 minutes prior to blood collection. Blood samples were collected by clean plastic syringes. Plastic tubes (TERUMO, Tokyo, Japan) containing 3.13% sodium citrate in a ratio of 1:9, were used to collect blood and immediately mixed. Platelet rich plasma (PRP) was prepared by centrifuging at 1000 rpm for 10 minutes. The plasma layer was examined for red cells, and precautions were followed to avoid red and white blood cells during transfer. With the use of plastic pipette, the PRP was carefully transferred into a polystyrene plastic tube (BD Falcon, Becton Dickinson and Company, Freanklin Lakes NJ, USA) and kept at room temperature. Platelet poor plasma (PPP) was prepared by centrifuging the remaining blood at 3000 rpm for 10 minutes. The platelet counts were within $20\text{-}40\times10^4$/ul. Platelet aggregation was measured by transparency detection of the PRP sample in comparison with each relevant PPP using platelet aggregation analyzer (MCM hematracer 313, PAM-12C; MC Medical Inc., Tokyo, Japan). The aggregation response was traced for one minute before and seven minutes after the addition of 20 ul of vehicle, adenosine diphosphate (ADP) (Arkray Inc., Kyoto, Japan), or AVP (Sigma Chemical Co., St. Louis, USA) into 200ul of PRP. The final concentrations of ADP and AVP were 5 uM and 1 nM, respectively. The results of platelet aggregation were always interpreted against ADP as the positive control and vehicle as the negative control. Samples that showed insufficient aggregation to ADP were not included in the study. No samples showed an aggregation response to the vehicle. All reagents for the platelet aggregation were freshly reconstituted. The analysis was always performed within 30-60 minutes by the same investigator after the PPP was prepared.

SNPs in the V1aR Gene

Our preliminary study identified 4 SNPs in the promoter region (−6951G/A, −4112A/T, −3860T/C, and −242C/T) of V1aR gene in 4 Japanese individuals by polymerase chain reaction (PCR) Direct Sequencing using Big Dye Terminator Method (ABI PRISM 310 Genetic Analyzer;

Applied Biosystems, CA, USA). GenBank sequence database, ACCESSION AF208541 and NM_000706 were used as reference sequence for searching SNPs in promoter and exonic regions of V1aR gene. No other polymorphisms were detected in exons and 4 SNPs in the promoter region of V1aR gene were confirmed by re-sequencing.

Determination of SNPs in the V1aR gene

Genomic DNA was extracted from peripheral blood using commercially available DNA extraction kit (SMI TEST; Sumitomo Bio-Medical, Kashima, Japan). Primers, for genotyping of 4 SNPs were designed in our laboratory from GenBank sequence database (ACCESSION AF208541). Primers sequences and locations are shown in Table 1.

−6951 G/A

PCR-restriction fragment length polymorphism (PCR-RFLP) was performed. Briefly, PCR was performed using primers designed for −6951G/A (Table 1) in a 25 μl volume containing 1X PCR buffer including 15mM $MgCl_2$, 2.5 mM each dNTP Mixture, 0.75 U TaKaRa Taq™ (Takara Bio Inc., Otsu Shiga, Japan), and 200 ng of genomic DNA. The cycling conditions were 94℃ for 3 min; 30 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 1 min; and 72℃ for 5 min. The PCR products were digested with 10 units of Bfa I (New England Biolabs, Beverly, MA, USA) in 1X NEBuffer 4, at 37℃ for 3 h. The products were subjected to electrophoresis on 2% agarose gel (SeaKem® LE Agarose, Cambrex Bio Science, Inc., Rockland, ME, USA) and stained with ethidium bromide. The G allele was detected as bands of 575 and 136 bp, while the A allele was detected as bands of 484, 136, and 91 bp.

−4112 A/T

PCR with allele-specific primers (PCR-ASA) was applied. PCR was done using a wild forward primer (A), a mutant forward primer (T) and a common reverse primer (Table 1). A 25 μl volume reaction mixture contained 1X PCR buffer including 15mM $MgCl_2$, 25 mM each dNTP Mixture, 25 mM $MgCl_2$, 1.25 U TaKaRa TaqTM (Takara Bio Inc., Otsu Shiga, Japan), and 100 ng of genomic DNA. The cycling conditions were

Table 1 Primers used to detect four polymorphisms in the promoter region of vasopressin V1a receptor gene

Variants	Direction	Location	Sequence
−6951 G/A	F	1714-1733	5′-TGT TGC TTC AAA CTT GGC AG-3′
	R	2424-2400	5′-TGT CTA AAT TTG ATC ATC AAG ACT C-3′
−4112 A/T	F-w	4766-4781	5′-GAA GCG GGA GGT TTC A-3′
	F-m	4766-4781	5′-GAA GCG GGA GGT TTC T-3′
	R	4909-4890	5′-AGA TTC ACT GAG CCA GAC TA-3′
−3860 T/C	F	4873-4899	5′-AAT AGT CAC TTA TGC CTT AGT CTG GCT-3′
	R	5069-5041	5′-ACT CTT AGT CTG TAA CAG AGA ATG CTC CT-3′
−242 C/T	F	8518-8539	5′-GGA GTA GGC AAC CAG CAG TCT T-3′
	R	8675-8652	5′-CCT GCT CTG CCT TTT TTC AAC TGC-3′

F: forward; R: reverse; F-w: forward-wild; F-m: forward-mutant.

94℃ for 3 min; 25 cycles of 94℃ for 30 s, 55℃ for 30 s, and 65℃ for 1 min; and 65℃ for 5 min. The products were separated on 2% agarose gel (SeaKem® LE Agarose, Cambrex Bio Science, Inc., Rockland, ME, USA) and visualized by ethidium bromide staining: a 144bp single band with the wild forward primer was wild type whereas single band 144 bp with mutant forward primer was mutant type.

−3860 T/C

PCR-RFLP was applied. The PCR conditions were the same as those for SNP −6951G/A, except that the annealing temperature was 53℃ and total cycle was 35. The PCR products were digested with 5 units of Tsp509 I (New England Biolabs, Beverly, MA, USA) in 1X NEBuffer 1, at 65℃ for 3 h. Electrophoresis on 2% agarose gel (SeaKem® LE Agarose, Cambrex Bio Science, Inc., Rockland, ME, USA) and staining with ethidium bromide detected T allele as bands of 158 and 39 bp, while the C allele as band of 197 bp.

−242 C/T

−242 C/T was also detected by PCR-RFLP. The PCR conditions were the same as those for SNP −3860T/C, except that the annealing temperature was 60℃. The PCR products were digested with 6 units of Pst I (Takara Bio Inc., Otsu Shiga, Japan) in 1X Buffer H, at 37℃ for 3 h. Electrophoresis on 4% NuSeive agarose gel (NuSieve® GTG® Agarose, Cambrex Bio Science, Inc., Rockland, ME, USA) and staining with ethidium bromide detected C allele as band of 157, while the T allele were detected as bands of 136 and 21 bp.

These genotype screenings always included previously determined, sequencing confirmed homozygous and heterozygous subjects for 4 SNPs as controls to ensure correct genotyping. Moreover, some of our randomly selected studied subjects from each group were sequenced to confirm genotypes, resulting always accurate.

Haplotype estimation and statistical analysis

Haplotypes were determined based on the genotype data of 4 SNPs of V1aR gene. The expectation/maximization (EM) algorithm[32] was applied to estimate the frequency of the haplotype. Haplotyps, Hardy-Weinberg equilibrium and linkage disequilibrium coefficient (D′) were estimated by using Helix Tree Genetic Analysis Software (version 4.0.3) [www.goldenhelix.com/em_algorithm.html].

Data are expressed as means ± SD unless otherwise stated. Basic continuous characteristics between control subjects and cases were analyzed by Student's t-test. χ^2 analysis with Yate's correction was applied to examine the differences in genotypes, alleles, haplotypes, as well as haplotype combinations between the groups; odds ratios (ORs) and their 95% confidence intervals (CIs) had been calculated. To asses the independent contribution of confounding factors, logistic regression analysis was performed with the SPSS package (SPSS Inc.). The relationship between responses to AVP and those to ADP was evaluated using Pearson's correlation analysis. The differences in platelet responses between groups was evaluated by one-way analysis of variance (ANOVA). Fisher's exact test was applied to examine the differences in genotypes between AVP responder and non-responder groups. P value of less than 0.05 was considered statistically significant.

Results

V1aR SNP associated with essential hypertension in nonobese individuals

Both SBP and DBP were higher in HT than in HS. 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 (Table 2).

Genotype distributions at each of 4 polymorphic sites of V1aR gene were in the Hardy-Weinberg equilibrium in all subjects (−6951G/A: χ^2 = 1.82, P = 0.17; −4112 A/T: χ^2 = 0.67, P = 0.41; −3860 T/C: χ^2 = 0.05, P = 0.82; −242 C/T: χ^2 = 0.55, P = 0.46).

The overall distribution of genotypes and alleles of 4 SNPs did not differ significantly between HS and total HT (data not shown). However, when HT were stratified according to obesity (BMI ≥ 25.0 kg/㎡), there was significant association between genotypes and alleles of the V1aR polymorphisms and the risk of hypertension. Among HT, there were 201 patients with BMI < 25.0 kg/㎡ and regarded as nonobese hypertensives (NOB-HT) [age (mean ± SD): 62.0 ± 9.8 yrs; sex (male/female): 82/119; BMI (mean ± SD): 22.4 ± 1.8 kg/㎡]; while there were 164 patients with BMI ≥ 25.0 kg/㎡ and regarded as obese hypertensives (OB-HT) [age (mean ± SD): 59.1 ± 9.2 yrs; sex (male/female): 61/103; BMI (mean ± SD): 27.8 ± 2.4 kg/㎡]. The prevalence of GA+AA genotype of SNP −6951 was significantly higher in NOB-HT compared with HS (P = 0.016; OR, 1.65; CI, 1.11−2.44). The frequency of minor A allele of SNP −6951 was also significantly higher in NOB-HT compared with HS (P = 0.019; OR, 1.51; CI, 1.10−2.10). Genotypes and alleles of SNP −6951G/A did not show any significant differences between HS and OB-HT. With the exception of significant difference of allele frequency for −3860T/C SNP (T allele vs. C allele: P = 0.037; OR, 1.33; CI, 1.0−1.74), genotype and allele distributions of 3 other SNPs of V1aR (−4112 A/T, −3860 T/C, and

Table 2 Clinical characteristics of healthy subjects (HS) and hypertensives (HT)

	HS (n = 255)	HT (n = 365)	P
Age, yrs	59.1 ± 10.5	60.7 ± 9.7	NS
Sex, M/F	81/174	143/222	NS
BMI, kg/㎡	21.6 ± 2.0	24.8 ± 3.4	<0.001
SBP, mmHg	120.2 ± 11.3	150.9 ± 20.0	<0.001
DBP, mmHg	72.5 ± 8.8	89.2 ± 12.2	<0.001
T-Chol, mg/dl	199.3 ± 31.4	205.5 ± 33.9	0.021
HDL, mg/dl	65.7 ± 15.5	61.2 ± 15.7	<0.001

Values are means ± SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; NS, not significant. Healthy subjects had SBP<140 and DBP<90, BMI<25.0 kg/㎡; HS had no diabetes, renal and hepatic disorders. Hypertensives had SBP ≥140 or DBP≥90 or were under antihypertensive treatment.

−242 C/T) also did not show any significant differences among all these groups (Tables 3).

To confirm that SNP −6951 G/A was independently associated with hypertension in nonobese individuals, we performed logistic regression analysis in subjects including HS and NOB-HT. It revealed that the significant association of SNP −6951 G/A with hypertension in the nonobese (P = 0.028) remained after adjustment for confounding factors (Table 4), and the calculated odds ratio was 1.57 (95% confidence interval: 1.05−2.35).

In all subjects, these 4 SNPs were

Table 3 Comparison of genotype and allele frequencies of V1aR gene SNPs between healthy subjects (HS) and hypertensives (HT) stratified based on obesity (BMI ≥ 25 kg/m²)

SNPs	Genotypes /Alleles	HS	HT			
			NOB-HT		OB-HT	
		(n = 255)	(n = 201)	*P*	(n = 164)	*P*
−6951G/A						
	GG	182 (71.0)	121 (60.0)		115 (70.0)	
	GA+AA	73 (29.0)	80 (40.0)	0.016	49 (30.0)	0.87
	G	428 (84.0)	312 (78.0)		272 (83.0)	
	A	82 (16.0)	90 (22.0)	0.019	56 (17.0)	0.78
−4112A/T						
	AA	179 (70.0)	150 (75.0)		125 (76.0)	
	AT+TT	76 (30.0)	51 (25.0)	0.35	39 (24.0)	0.22
	A	428 (84.0)	350 (87.0)		286 (87.0)	
	T	82 (16.0)	52 (13.0)	0.22	42 (13.0)	0.23
−3860T/C						
	TT	96 (38.0)	61 (30.0)		61 (37.0)	
	TC+CC	159 (62.0)	140 (70.0)	0.13	103 (63.0)	0.99
	T	314 (62.0)	219 (54.0)		200 (61.0)	
	C	196 (38.0)	183 (46.0)	0.037	128 (39.0)	0.92
−242C/T						
	CC	192 (75.0)	158 (79.0)		128 (78.0)	
	CT+TT	63 (25.0)	43 (21.0)	0.47	36 (22.0)	0.60
	C	444 (87.0)	357 (89.0)		290 (88.0)	
	T	66 (13.0)	45 (11.0)	0.48	38 (12.0)	0.64

Values are actual counts (%). V1aR, vasopressin V1a receptor; SNPs, single nucleotide polymorphisms; NOB-HT, nonobese hypertensive (BMI < 25.0 kg/m²); OB-HT, obese hypertensive (BMI ≥ 25.0 kg/m²).

Table 4 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.

in strong and highly significant linkage disequilibrium with each other (−6951 vs. −4112: D′ = 0.98, $P < 0.001$; −6951 vs. −3860: D′ = 0.99, $P < 0.001$; −6951 vs. −242: D′ = 0.85, $P < 0.001$; −4112 vs. −3860: D′ = 0.97, $P < 0.001$; −4112 vs. −242: D′ = 0.98, $P < 0.001$; −3860 vs. −242: D′ = 0.99, $P < 0.001$).

We determined 12 different haplotypes based on 4 SNPs of V1aR and consequently designated H1 to H12. However, haplotypes with rare frequencies (≤1.0%) in cases of both HS and HT were not considered for analysis. The distributions of major haplotypes (H1-H5) between the HS and NOB-HT group are shown in Table 5. While haplotype distributions between HS and HT had no significant differences (data not shown), comparing distribution between HS and NOB-HT revealed significantly higher frequency of H3 haplotype in NOB-HT ($P = 0.048$) (Table 5) that include the −6951 variant. Analysis of haplotype combinations (diplotype) based on the evaluated haplotypes did not show any significant differences between healthy subjects and NOB-HT (Table 5).

V1aR SNP associated with type 2 diabetes mellitus; V1aR SNP had an effect on HbA_{1C} status

Genotype distributions at each of 4 polymorphic sites of V1aR gene were in the Hardy-Weinberg equilibrium in all subjects (−6951G/A: $\chi^2 = 1.23$, $P = 0.27$; −4112 A/T: $\chi^2 = 1.16$, $P = 0.28$; −3860 T/C: $\chi^2 = 0.32$,

Table 5 Distribution of haplotypes and haplotype combinations in healthy subjects (HS) and nonobese hypertensives (NOB-HT)

		HS	NOB-HT	*P*	OR (95%CI)
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)
Haplotype Combinations					
H1/H1	GG-AA-TT-CC	50 (19.6%)	37 (18.4%)	0.84	1.08 (0.67-1.73)
H1/H2	GG-AA-TC-CC	59 (23.1%)	36 (17.9%)	0.21	1.38 (0.87-2.19)
H1/H3	GA-AA-TC-CC	32 (12.5%)	34 (16.9%)	0.24	0.70 (0.42-1.18)
H1/H4	GG-AT-TT-CT	29 (11.4%)	19 (9.4%)	0.61	1.23 (0.67-2.26)
H1/H5	GG-AT-TT-CC	10 (3.9%)	3 (1.5%)	0.21	2.69 (0.73-9.90)
H2/H2	GG-AA-CC-CC	10 (3.9%)	15 (7.5%)	0.15	0.51 (0.22-1.15)
H2/H3	GA-AA-CC-CC	18 (7.1%)	17 (8.5%)	0.70	0.82 (0.41-1.64)
H3/H3	AA-AA-CC-CC	9 (3.5%)	11 (5.5%)	0.44	0.63 (0.26-1.55)

Values in case of haplotype frequencies are counts (fraction). Values in case of haplotype combinations are counts (%). 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. Haplotype combinations were based on the haplotypes with frequencies of > 1.0% in HS and NOB-HT.
OR, odds ratio; CI, confidence interval.

P = 0.57; −242 C/T: χ^2 = 0.29, P = 0.59).

The genotype frequency for −6951G/A variant of the V1aR gene in T2DM was 65.0% for GG and 35.0% for GA+AA. In CS, the genotype frequency was 76.0% for GG and 24.0% for GA+AA. There was a significant difference (P = 0.048; OR, 1.60; CI, 1.03−2.5) in −6951G/A genotype distribution between T2DM and CS groups (Table 6). Allele frequencies of G and A were 80.0% and 20.0% in T2DM, whereas 85.0% and 15.0% in CS group. Although A allele of −6951 SNP was found to be more frequent in T2DM, this was not significant (Table 6). Genotype and allele distributions of 3 other SNPs of V1aR (−4112 A/T, −3860 T/C, and −242 C/T) did not show any significant differences between the T2DM and CS groups (Tables 6). To confirm that SNP −6951 G/A was independently associated with type 2 DM, we performed logistic regression analysis in subjects including CS and T2DM. It revealed that the significant association of SNP −6951 G/A with type 2 DM (P = 0.04) remained after adjustment for after adjustment for age and sex, and the calculated odds ratio was 1.59 (95% confidence interval: 1.02−2.49).

In young healthy subjects, HbA_{1C} levels

Table 6 Comparison of genotype and allele frequencies of V1aR SNPs between control subjects (CS) and patients with type 2 diabetes mellitus (T2DM)

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)
−4112A/T					
	AA	135) (72.0)	131 (70.0)		
	AT+TT	53 (28.0)	55 (30.0)	0.86	1.01 (0.68-1.68)
	A	320 (85.0)	314 (84.0)		
	T	56 (15.0)	58 (16.0)	0.87	1.05 (0.71-1.57)
−3860T/C					
	TT	72 (38.0)	66 (35.0)		
	TC+CC	116 (62.0)	120 (65.0)	0.65	1.13 (0.74-1.7)
	T	234) (62.0)	216 (58.0)		
	C	142 (38.0)	156 (42.0)	0.28	1.20 (0.89-1.6)
−242C/T					
	CC	143 (76.0)	140 (75.0)		
	CT+TT	45 (24.0)	46 (25.0)	0.95	1.0 (0.65-1.67)
	C	328 (87.0)	324 (87.0)		
	T	48 (13.0)	48 (13.0)	0.96	1.0 (0.66-1.6)

Values are actual counts (%). V1aR, vasopressin V1a receptor; SNPs, single nucleotide polymorphisms; OR, odds ratio; CI, confidence interval.

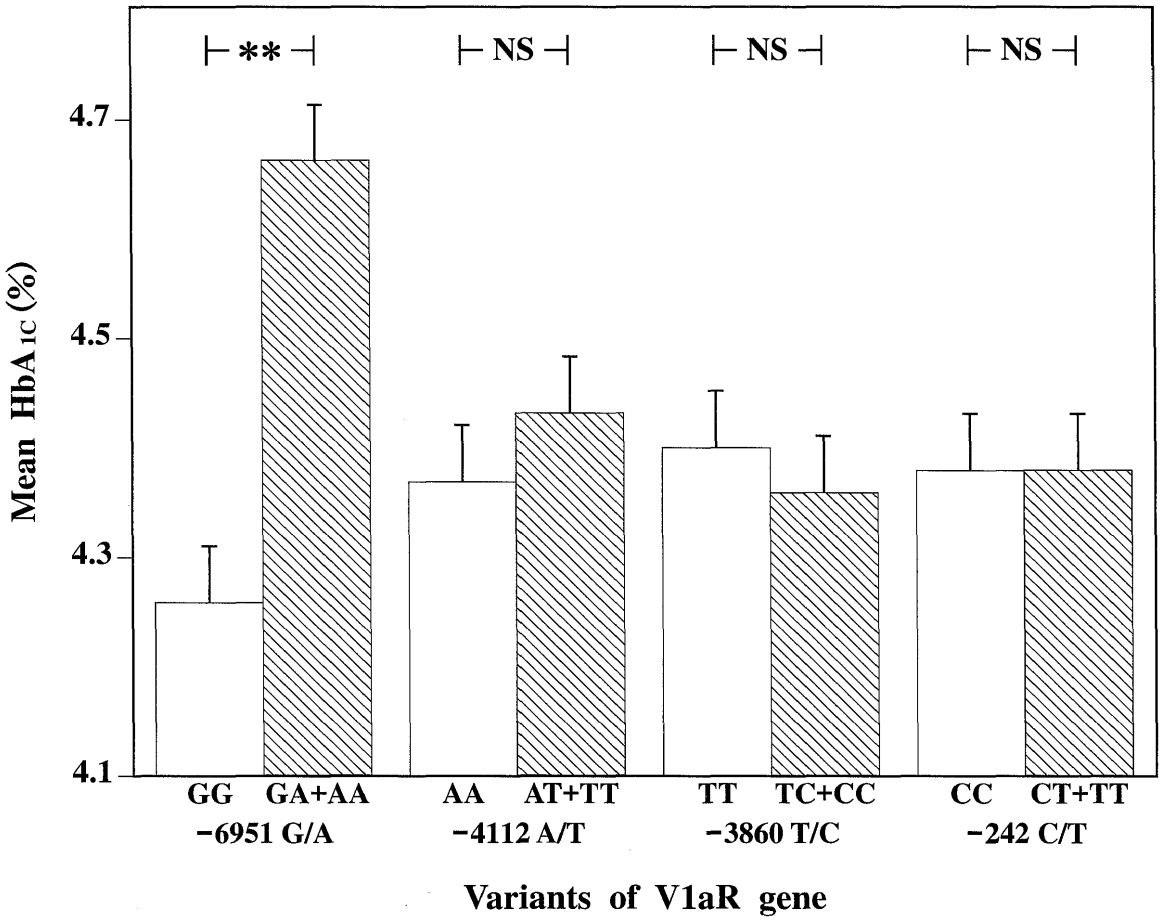

Figure 1 HbA_{1C} status and V1aR SNPs in young healthy subjects.
** $P < 0.001$; NS, not significant.

are shown in Figure 1 based on the findings of 4 SNPs of V1aR gene. GA+AA carriers of SNP −6951G/A had significantly ($P < 0.001$) higher HbA_{1C} (4.66 ± 0.13) compared with GG carriers (4.26 ± 0.24). However, HbA_{1C} did not show any significant differences for each of 3 other SNPs of V1aR.

In all subjects, these 4 SNPs were in strong linkage disequilibrium with each other (−6951 vs. −4112: D′ = 0.69, $P < 0.01$; −6951 vs. −3860: D′ = 0.98, $P < 0.001$; −6951 vs. −242: D′ = 0.98, $P < 0.001$; −4112 vs. −3860: D′ = 0.69, $P < 0.001$; −4112 vs. −242: D′ = 0.83, $P < 0.001$; −3860 vs. −242: D′ = 0.88, $P < 0.001$).

The distributions of major haplotypes (H1-H5) showed H1 (g-a-t-c) to be more frequent in CS than T2DM. However, the difference did not reach the significance level. Analysis of haplotype combinations based on the evaluated haplotypes showed that H2/H3 heterozygote was more frequent in T2DM than in CS (H2/H3: 8.6% versus 3.7%; $P = 0.08$; OR, 2.43; CI, 1.0−5.90). On the other hand, the H1/H2 heterozygote appeared more frequently in CS than in T2DM (H1/H2: 22.0% versus 14.5%; $P = 0.07$; OR, 0.59; CI, 0.348−1.0). However, the difference was not significant.

Although haplotype as well as haplotype combinations did not show any significant differences between CS and T2DM, haplotype

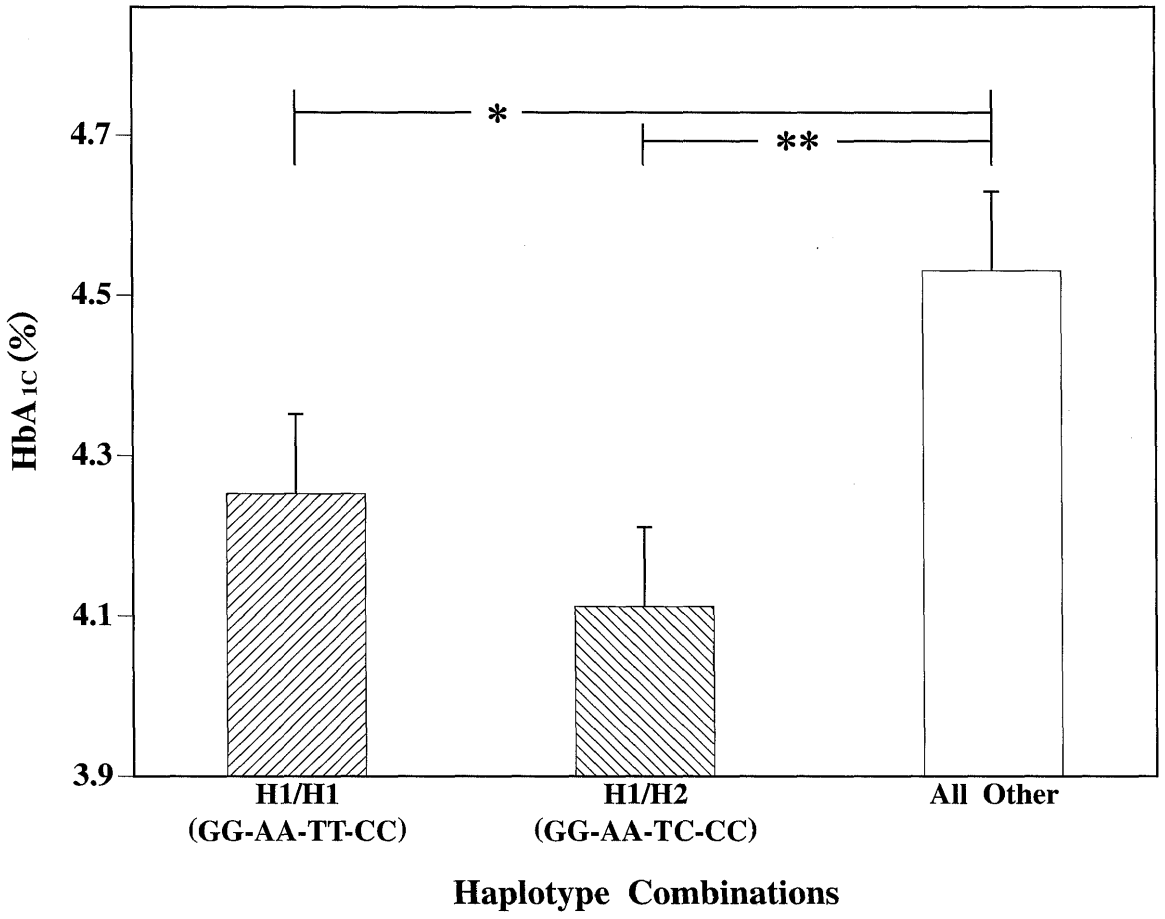

Figure 2 HbA_{1C} status and haplotype combinations based on 4 SNPs of V1aR gene in young healthy subjects.
* $P < 0.01$; ** $P < 0.001$.

combinations showed significant relation with the glycemic status. 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 2).

AVP and ADP might share a common pathway in inducing platelet aggregation; No association of V1aR gene 4 SNPs with platelet vasopressin responsiveness

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%. Seventeen of 33 subjects showed more than 60% of maximum platelet aggregtation by AVP and were categorized as responders to AVP, whereas 16 were classified as non-responders because they had less than 30% aggregation. There was a significant correlation ($r = 0.59$; $P < 0.001$) between responses to AVP and those to ADP (data not shown).

When platelet aggregations in response to AVP were compared among the groups defined by each of the 4 variants of V1aR gene, no significant association was found between the genotype and the platelet response ($P = 0.77$ for −6951G/A; $P = 0.65$ for −4112A/T; $P = 0.52$ for −3860T/C and

P = 0.55 for −242C/T) (data not shown).

We compared genotype distributions of 4 variants in V1aR between the responder group and the non-responder group. However, there was no significant association (P = 0.52 for −6951G/A; P = 0.32 for −4112A/T; P = 0.57 for −3860T/C and P = 0.68 for −242C/T) (data not shown).

Discussion

In the present study, none of the genotypes and alleles of 4 V1aR SNPs showed any significant differences between HS and HT. However, after splitting HT based on obesity, prevalence of GA+AA genotype and frequency of A allele for the SNP −6951G/A were significantly greater in NOB-HT than in HS. Multiple logistic regression analysis showed that the SNP −6951G/A is an independent risk factor for hypertension without obesity. On the other hand, there was no association of any genotypes or alleles for 3 other SNPs of the V1aR gene, except for −3860T/C allele distribution which was significantly higher in NOB-HT. However, analysis of the distribution of haplotype, defined by 4 SNPs of the V1aR gene, demonstrated that one haplotype named H3 (a-a-c-c) including −6951G/A variation was more frequent in NOB-HT than in HS. These results suggest that V1aR gene variations may confer susceptibility to hypertension independently of obesity.

Essential hypertension is a heterogeneous disorder where several overlapping subsets of mechanisms may underlie the condition. It has become evident that clinically defined subtypes of hypertension, such as obesity-associated hypertension, may be genetically distinct from hypertension in nonobese individuals. Recently, 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. Indeed, metabolic syndrome, a condition characterized by abdominal obesity that increases the risk of high blood pressure, high cholesterol and diabetes, is widely accepted as an independent clinical entity. Therefore, it seems quite reasonable for us to stratify hypertensives according to their BMI. The HYPERGENE studies already implicated the association of angiotensinogen gene in the development of hypertension in individuals with BMI < 27 kg/m^2[34,35]. Hepatocyte growth factor gene polymorphism was reported to associate with susceptibility to essential hypertension in lean subjects[36]. The present results also suggest that AVP V1aR gene could be one of candidate genes for hypertension in nonobese Japanese individuals. Our present results confirm the view, from the single gene level, that hypertension in obese and overweight subjects may be, at least in part, genetically distinct from that in nonobese individuals.

Dissecting hypertensives by ethnicity has also been shown to reduce the level of genetic heterogeneity. Morris[37] stated that Pausova *et al*[33] succeeded to elevate the degree of genetic homogeneity by recruiting isolated population from the geographically remote French-Canadian Saguenay/Lac-St-Jean region of Quebec. Thus, despite a relatively small number of families being studied, a significant locus has emerged. The results by Pausova *et al*[33] are in contrast with the 2 largest genome-wide 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, all subjects were native Japanese residents in the rural district, Aomori prefecture, in a traditional, conservative, and agriculture-based lifestyle. Their predecessors lived there over the past several centuries. Therefore, it is likely that our relatively small, isolated population has also been spared the level of genetic "noise" present in mixed populations elsewhere.

The role of AVP and its vascular effector V1aR in blood pressure regulation has been well documented over the past few decades. However, it has been obscure whether AVP and its receptors are really candidate genes for essential hypertension. Thibonnier *et al*[26] challenged both case-control and linkage studies using several microsatellite polymorphisms through the whole V1aR gene in French Caucasians with negative results. They did not dissect hypertensives by obesity or ethnicity. Our present results are not inconsistent with their results, because we found no association between V1aR gene SNPs and hypertension as a whole. Although none of the microsatellite polymorphisms were found in our Japanese subjects, it appears to be an attractive approach whether those variations have any association with nonobese hypertension in French population.

The present study seems to be the first to evaluate the implications of V1aR gene polymorphisms on type 2 DM. AVP action on glycogenolysis is mediated by hepatic V1aR. The high abundance in the liver enabled cloning and expression of a rat V1aR[40]. The occupancy of this receptor stimulates several metabolic pathways including glycogenolysis, gluconeogenesis, and ureagenesis[41,42]. Down regulation of V1aR has been demonstrated in DM in the rats[43,44]. In another study using genetically diabetic (db/db) mice, AVP was found to regulate hepatic V1aR expression in the progress of genetic DM[45]. Our study found a significant association of −6951G/A SNP of V1aR gene with type 2 DM. Logistic regression also showed the significant association of the SNP −6951G/A with type 2 DM after adjustment for age and sex. GA+AA carrier of this SNP also significantly associated with the glycemic status as revealed by the HbA_{1C} in young healthy subjects. A couple of haplotype combinations generated from haplotypes of 4 SNPs of V1aR gene also showed characteristic features between CS and T2DM groups. Couples of haplotype combinations also were in good agreement in terms of glycemic status in young healthy subjects. These results indicate the importance of −6951G/A SNP as a genetic marker of type 2 DM and haplotypes in predicting type 2 DM.

AVP has been reported to stimulate platelet aggregation by platelet V1aR. However, there is heterogeneity of the aggregation responses of human platelets to AVP[18]. This study was the first to investigate the implication of V1aR SNPs in recognizing individuals who have enhanced platelet activation and consequently are at risk for cardiovascular disease. We confirmed that platelet aggregation response to AVP varies among individuals. Platelet aggregation was also evaluated in response to other physiological agonist ADP. We found a significant correlation between responses to AVP and those to ADP. This study is the first to compare platelet aggregation in response to ADP and AVP and significant correlation between these suggests that they may share a common pathway in inducing platelet aggregation. AVP action through the V1a receptor is mediated by activating phopholipase C, via Gq/11[20]. Several studies have demonstrated that the activation of phospholipase C via Gq is indispensable for ADP-induced platelet aggregation[46,47].

There were no significant association in the AVP-induced aggregation between the subjects with and without variant alleles of each 4 SNPs. The genotype distribution of 4 SNPs were almost identical between AVP responders and nonresponders. These results suggest that our identified 4 promoter SNPs of V1aR gene may not be useful as genetic markers for platelet aggregation heterogeneity. It appears that genes involved in the post-receptor signal pathway, such as protein kinase C,[11] may play a dominant role in the diversity of AVP action on platelets.

It seems also reasonable that there might be other polymorphisms of V1aR gene as a more useful marker than the SNPs studied in the present study.

The sample size for studying the association of V1aR SNPs with the glycemic status and platelet aggregation responses to AVP was small. However, significant association of −6951G/A SNP with the increased HbA_{1C} level demonstrates concordance with the finding of the association of the same SNP with type 2 DM mellitus. The lack of association of V1aR SNPs with platelet aggregation responses to AVP might be also due to small sample size. Therefore, further larger study will be needed to confirm the effect of V1aR SNPs on glycemic status as well as platelet responsiveness to AVP.

It is well known that V1aR gene is expressed constitutively. The 5′-flanking region of rat V1aR gene is reported to have neither a TATA nor a CCAAT box but have features of promoters seen in housekeeping genes[48]. Young *et al* found that the promoter of V1aR gene plays a key role in regulating its expression in rodents, because an insertion/deletion variation in the promoter determines the expression levels and expression patterns within the brain in close conjunction with affiliative behavior in rodents[49]. However, there is little evidence available whether those findings are applicable to humans or not. Among the 4 SNPs in the present study, 3 SNPs are located within some transcription factor response elements according to our computational analysis. Namely, −4112A/T is within the element for DeltaE, one of NOTCH1 lacking a major portion of the extracellular domain, −3860T/C for nuclear factor-1, and −242C/T for heat shock transcription factor, respectively. Therefore, these SNPs might affect the transcriptional activity of V1aR gene. SNP −6951 might have role in regulating the expression of V1aR gene. Investigating V1aR promoter activity in regulating its expression in appropriate cell line using reporter gene construct containing the SNP −6951 would clarify the functional relevance of this SNP. Recently, micro RNAs, small non-coding endogenous RNA molecules, have been implicated to regulate gene expression by interacting with target mRNAs at specific sites to induce cleavage of the message or inhibit translation. There are several micro RNAs identified showing target site in the V1aR gene. In this context, −6951 site might code micro RNA inhibiting translation of the V1aR gene. Therefore, it would be interesting to investigate role of micro RNAs in V1aR gene expression.

In conclusion, the present study demonstrates significant associations of the V1aR SNP −6951 with NOB-HT, T2DM as well as glycemic status in healthy subjects. The haplotype H3 including −6951 variation reveals significantly greater frequency in NOB-HT. Distribution of haplotype combinations were in good agreement with T2DM and glycemic status in healthy subjects. Therefore, the presence of V1aR gene variations might lead to an increased

risk of hypertension in nonobese individuals and type 2 DM. However, 4 SNPs of V1aR might not be useful as genetic marker for platelet aggregation heterogeneity. Future investigations will be needed in this and other populations to elucidate the functional implications of the genetic associations that have been observed in this study.

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