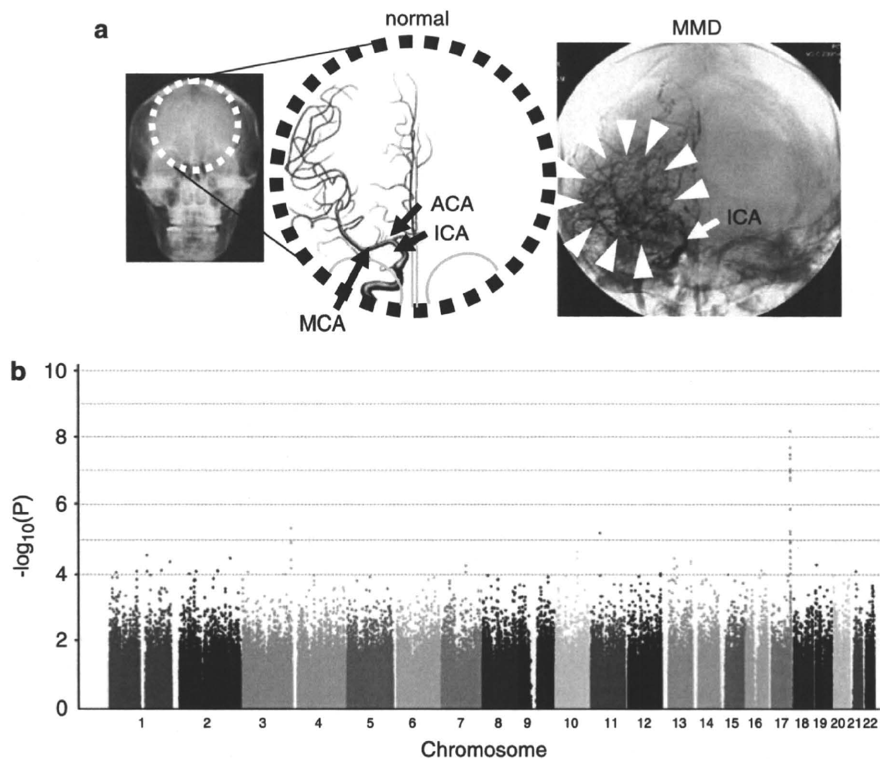

Figure 1 **(a)** Abnormal brain vessels in MMD. The dotted circle indicates the X-ray field of cerebral angiography (left panel). Normal structures of the right internal carotid artery (ICA), anterior cerebral artery (ACA) and middle cerebral artery (MCA) are illustrated (middle panel). The arrowheads indicate abnormal collateral vessels appearing like a puff of smoke in the angiogram of an individual with MMD (right panel). Note that ACA and MCA are barely visible, because of the occlusion of the terminal portion of the ICA. **(b)** Manhattan plot of the 785 720 SNPs used in the genome-wide association analysis of MMD patients. Note that the SNPs in the 17q25-ter region reach a significance of $P<10^{-8}$.

MATERIALS AND METHODS

Affected individuals

Genomic DNA was extracted from blood and/or saliva samples obtained from members of the families with MMD (Supplementary Figure 1), MMD patients with no family history and control subjects. All of the subjects were Japanese. MMD was diagnosed on the basis of guidelines established by the Research Committee on Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare of Japan. This study was approved by the Ethics Committee of Tohoku University School of Medicine. Total RNA samples were purified from leukocytes using an RNeasy mini kit (Qiagen, Hilden, Germany) and used as templates for cDNA synthesis with an Oligo $(dT)_{20}$ primer and SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Linkage analysis

For the linkage analysis, DNA samples were genotyped for 36 microsatellite markers within five previously reported MMD loci using the ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Pedigrees and haplotypes were constructed with the Cyrillic version 2.1 software (Oxfordshire, UK). Multipoint analyses were conducted using the GENEHUNTER 2 software (http://www.broadinstitute.org/ftp/distribution/software/genehunter/). Statistical analysis was performed with SPSS version 14.0J (SPSS, Tokyo, Japan).

Genome-wide and locus-specific association studies

A genome-wide association study was performed using a group of 72 MMD patients, which consisted of 64 patients without a family history of MMD and 8 probands of MMD families. The Illumina Human Omni-Quad 1 chip (Illumina, San Diego, CA, USA) was used for genotyping, and single-nucleotide polymorphisms (SNPs) with a genotyping completion rate of 100% were used for further statistical analysis (785 720 out of 1 140 419 SNPs). Genotyping data from 45 healthy Japanese controls were obtained from the database at the International HapMap Project web site. The 785 720 SNPs were statistically analyzed using the PLINK software (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml). For a locus-specific association study, we used 63 DNA samples consisting of 58 non-familial MMD patients and 5 probands of MMD families. A total of 384 SNPs within chromosome 17q25-ter were genotyped (Supplementary Table 1), using the GoldenGate Assay and a custom SNP chip (Illumina). Genotyping data for 45 healthy Japanese were used as a control. Case–control single-marker analysis, haplotype frequency estimation and significance testing of differences in haplotype frequency were performed using the Haploview version 3.32 program (http://www.broad.mit.edu/mpg/haploview/).

Mutation detection

Mutational analyses of *RNF213* and *FLJ35220* were performed by PCR amplification of each coding exon and putative promoter regions, followed by direct sequencing. Genomic sequence data for the two genes were obtained from the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/) for design of exon-specific PCR primers. *RNF213* cDNA fragments were amplified from leukocyte mRNA for sequencing analysis. Sequencing of the PCR products was performed with the ABI BigDye Terminator Cycle Sequencing Reaction Kit using the ABI 310 Genetic Analyzer. Identified base changes were screened in control subjects. Statistical difference of the carrier frequency of each base change was estimated by Fisher's exact test (the MMD group vs the control group).

Quantitative PCR

MTC Multiple Tissue cDNA Panels (Clontech Laboratory, Madison, WI, USA) were the source of cDNAs from human cell lines, adult and fetal tissues. Mononuclear cells and polymorphonuclear cells were isolated from the fresh peripheral blood of healthy human adults using Polymorphprep (Cosmo Bio,

Carlsbad, CA, USA). T and B cells were isolated from the fresh peripheral blood of healthy human adults using the autoMACS separator (Militeny Biotec, Bergisch Gladbach, Germany). Total RNA was isolated from these cells with the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. We reverse transcribed 100 ng samples of total RNA into cDNAs using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCRs were performed in a final volume of 20 µl using the FastStart TaqMan Probe Master (Rox) (Roche, Madison, WI, USA), 5 µl of cDNA, 10 µM of *RNF-* or *GAPDH*-specific primers and 10 µM of probes (Universal ProbeLibrary Probe #80 for *RNF213* and Roche Probe #60 for *GAPDH*). All reactions were performed in triplicate using the ABI 7500 Real-Time PCR system (Applied Biosystems). Cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Real-time PCR data were analyzed by the SDS version 1.2.1 software (Applied Biosystems). We evaluated the relative level of *RNF213* mRNA by determining the C_T value, the PCR cycle at which the reporter fluorescence exceeded the signal baseline. *GAPDH* mRNA was used as an internal reference for normalization of the quantitative expression values.

Multiplex PCR

MTC Multiple Tissue cDNA Panels (Clontech) were the source of human cell lines and cDNAs from human adult and fetal tissues. Multiplex PCRs were performed in a final volume of 20 µl using the Multiplex PCR Master Mix (Qiagen), 2 µl of cDNA, a 2 µM concentration of *RNF213* and a 10 µM concentration of *GAPDH*-specific primers. The samples were separated on a 2% agarose gel stained with ethidium bromide. Cycling conditions were 15 min at 94°C, followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C. For normalization of the expression levels, we used *GAPDH* as an internal reference for each sample.

In situ hybridization (ISH) analysis

Paraffin-embedded blocks and sections of mouse tissues for ISH were obtained from Genostaff (Tokyo, Japan). The mouse tissues were dissected, fixed with Tissue Fixative (Genostaff), embedded in paraffin by proprietary procedures (Genostaff) and sectioned at 6 µm. To generate anti-sense and sense RNA probes, a 521-bp DNA fragment corresponding to nucleotide positions 470–990 of mouse *Rnf213* (BC038025) was subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Hybridization was performed with digoxigenin-labeled RNA probes at concentrations of 300 ng ml^{-1} in Probe Diluent-1 (Genostaff) at 60 °C for 16 h. Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich, St Louis, MO, USA). The sections were counterstained with Kernechtrot stain solution (Mutoh, Tokyo, Japan), dehydrated and mounted with Malinol (Mutoh). For observation of *Rnf213* expression in activated lymphocytes, 10-week-old Balb/c mice were intraperitoneally injected with 100 µg of keyhole limpet hemocyanin and incomplete adjuvant and sacrificed in 2 weeks. The spleen of the mice was removed for Hematoxylin–eosin staining and ISH analyses.

RESULTS

Using 20 Japanese MMD families, we reevaluated the linkage mapped previously to five putative MMD loci. No locus with significant linkage, Lod score >3.0 or NPL score >4.0 was confirmed (Supplementary Figure 2). We conducted a genome-wide association study of 72 Japanese MMD cases. Single-marker allelic tests comparing the 72 MMD cases and 45 controls were performed for 785 720 SNPs using χ^2 statistics. These tests identified a single locus with a strong association with MMD ($P<10^{-8}$) on chromosome 17q25-ter (Figure 1b), which is in line with the latest mapping data of a MMD locus.[16] The SNP markers with $P<10^{-6}$ are listed in Table 1. To confirm this observation, we performed a locus-specific association study. A total of 384 SNP markers (Supplementary Table 1) were selected within the chromosome 17q25-ter region and genotyped in a set of 63 MMD cases and 45 controls. The SNP markers demonstrating a high association with MMD ($P<10^{-6}$) were clustered in a 151-kb region from base position 75 851 399–76 003 020 (SNP No.116–136 in

Table 1 A genome-wide association study of Japanese MMD patients and controls

	SNP	Chromosome	Base position	Gene	Risk allele/ non-risk allele	Risk allele frequency in MMD	Risk allele frequency in controls	χ^2	P-value	Odds ratio	95% confidence interval	
											Lower	Upper
1	rs11870849	17	76025668	*RNF213*	T/C	0.4792	0.1111	33.55	6.95E-09	7.36	3.532	15.34
2	rs6565681	17	75963089	*RNF213*	A/G	0.7361	0.3667	31.35	2.16E-08	4.819	2.733	8.489
3	rs7216493	17	75941953	*RNF213*	G/A	0.75	0.3889	30.39	3.53E-08	4.715	2.673	8.313
4	rs7217421	17	75850055	*RNF213*	A/G	0.6667	0.3	29.86	4.64E-08	4.666	2.642	8.237
5	rs12449863	17	75857806	*RNF213*	C/T	0.6667	0.3	29.86	4.64E-08	4.666	2.642	8.237
6	rs4890009	17	75926103	*RNF213*	G/A	0.8819	0.5778	28.5	9.38E-08	5.459	2.831	10.527
7	SNP17-75933731	17	75933731	*RNF213*	G/A	0.8819	0.5778	28.5	9.38E-08	5.458	2.831	10.527
8	rs7219131	17	75867365	*RNF213*	T/C	0.6667	0.3111	28.11	1.15E-07	4.429	2.517	7.794
9	rs6565677	17	75932037	*RNF213*	T/C	0.7431	0.3977	27.43	1.63E-07	4.378	2.483	7.722
10	rs4889848	17	75969256	*RNF213*	C/T	0.75	0.4111	26.99	2.05E-07	4.297	2.444	7.889
11	rs7224239	17	75969771	*RNF213*	A/G	0.8681	0.5667	26.99	2.05E-07	5.03	2.659	9.529

Abbreviations: MMD, moyamoya disease; SNP, single-nucleotide polymorphism.A genome-wide association study testing 1 140 419 SNPs on the Human Omni-Quad 1chip (Illumina, San Diego, CA, USA) was performed in 72 Japanese MMD cases. Single-marker allelic tests between the cases and controls were performed using χ^2 statistics for all markers. This table lists the 11 SNP markers with a significance of $P<10^{-6}$.

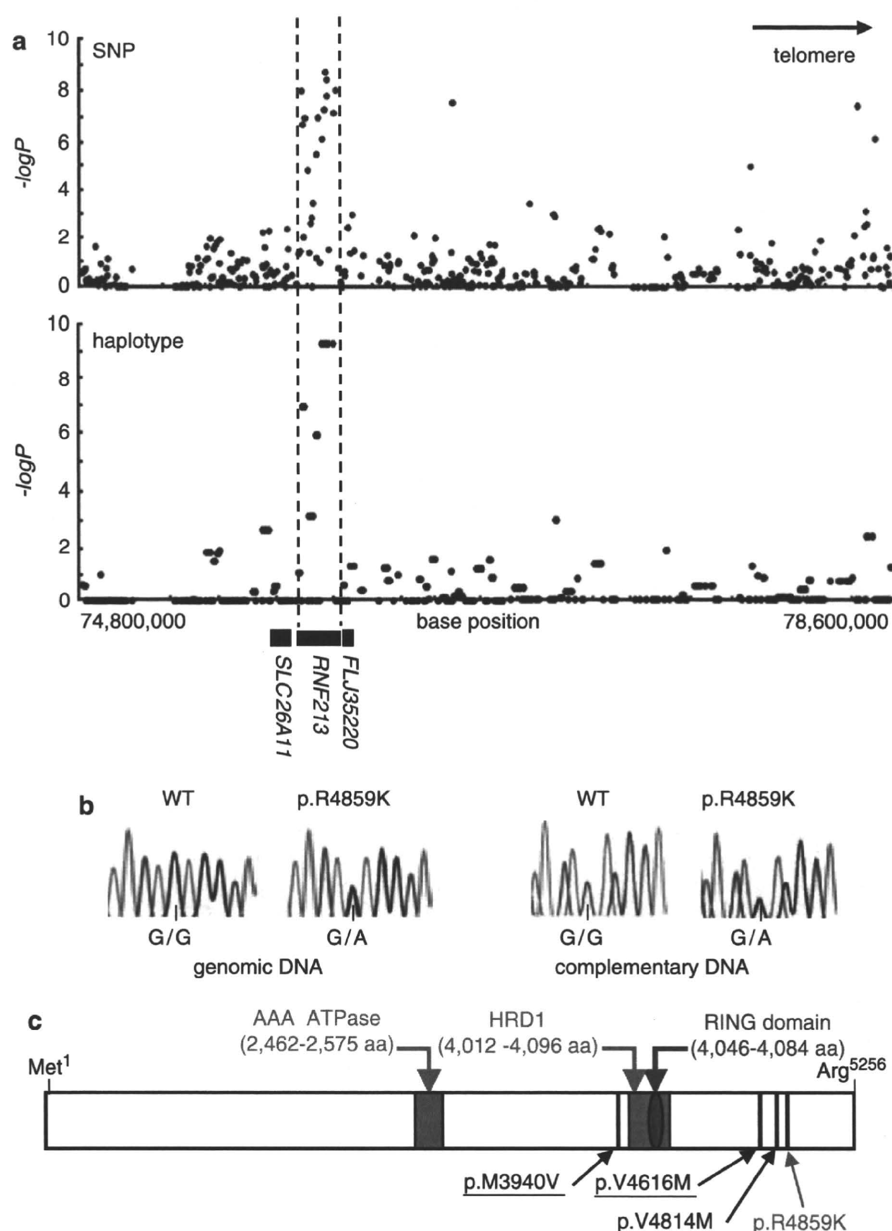

Figure 2 (**a**) Association analysis of 63 non-familial MMD cases and 45 control subjects. Statistical significance was evaluated by the χ^2-test. SNP markers with a strong association with MMD ($P<10^{-6}$) clustered in a 161-kb region (base position 75 851 399–76 012 838) indicated by two dotted lines (upper panel), which included the entire region of *RNF213* (lower panel). Haplotype analysis revealed a strong association ($P=5.3\times10^{-10}$) between MMD and a single haplotype located within *RNF213*. (**b**) Sequencing chromatograms of the identified MMD mutations. The left panel shows the sequences of an unaffected individual and a carrier of a p.R4859K heterozygous mutation. The right panel indicates the sequencing chromatograms of the leukocyte cDNA obtained from an unaffected individual and an individual with MMD who carries the p.R4859K mutation. Note that both wild-type and mutant alleles were expressed in leukocytes. (**c**) The structure of the RNF213 protein. The RNF213 protein contains three characteristic structures, the AAA-superfamily ATPase motif, the RING motif and the HMG-CoA reductase degradation motif. The positions of four mutations identified in MMD patients are underlined, including one prevalent mutation (red) and three private mutations (black).

Supplementary Table 1); this entire region was within the *RNF213* locus (Figure 2a). A single haplotype determined by seven SNPs (SNP Nos.130–136 in Supplementary Table 1) that resided in the 3′ region of *RNF213* was strongly associated with MMD onset ($P=5.3\times10^{-10}$). Analysis of the linkage disequilibrium block indicated that this haplotype was not in complete linkage disequilibrium with any other haplotype in this region (Supplementary Figure 3). These results strongly suggest that a founder mutation may exist in the 3′ part of *RNF213*.

Mutational analysis of the entire coding and promoter regions of *RNF213* and *FLJ35220*, a gene 3′ adjacent to *RNF213*, revealed that 19 of the 20 MMD families shared the same single base substitution, c.14576G>A, in exon 60 of *RNF213* (Figure 2b and Table 2). This nucleotide change causes an amino-acid substitution from arginine^{4859} to lysine^{4859} (p.R4859K). The p.R4859K mutation was identified in 46 of 63 non-familial MMD cases (73%), including 45 heterozygotes and a single homozygote (Table 3). Both the wild-type and the p.R4859K mutant alleles were co-expressed in leukocytes

Table 2 Nucleotide changes with amino-acid substitutions identified in the sequencing analysis of *RNF213* and *FLJ35220*

			Genotype (allele)				
Gene	*Exon*	*Nucleotide change[a] (amino-acid substitution)*	*Non-familial cases*	*Control subjects*	*P-value[b]*	*χ^2 (df=1)[c]*	*Odds ratio (95% CI)*
RNF213	29	c.7809C>A (p.D2603E)	2/63 (2/126)	15/381 (15/762)	0.77	0.09	0.80 (0.2–3.6)
RNF213	41	c.11818A>G (p.M3940V)	1/63 (1/126)	0/388 (0/776)	0.01	6.17	ND
RNF213	41	c.11891A>G (p.E3964G)	4/63 (4/126)	3/55 (4/110)	0.84	0.04	1.2 (0.3–5.5)
RNF213	52	c.13342G>A (p.A4448T)	4/63 (4/126)	2/53 (2/106)	0.53	0.39	1.7 (0.3–9.8)
RNF213	56	c.13846G>A (p.V4616M)	1/63 (1/126)	0/388 (0/776)	0.01	6.17	ND
RNF213	59	c.14440G>A (p.V4814M)	1/63 (1/126)	0/388 (0/776)	0.01	6.17	ND
RNF213	60	c.14576G>A (p.R4859K)	46/63 (47/126)	6/429 (6/858)	1.2×10^{-43}	298.1	190.8 (71.7–507.9)
FLJ35220		None					

Abbreviations: ND, not determined; SNP, single-nucleotide polymorphism.
[a]Nucleotide numbers of *RNF213* cDNA are counted from the A of the ATG initiator methionine codon (NCBI Reference sequence, NP_065965.4).
[b]*P*-values were calculated by Fisher's exact test.
[c]Genotypic distribution (carrier of the polymorphism vs non-carrier).

Table 3 Association of the p.R4859K (c.14576G>A) mutation with MMD

		Genotype		
	Total	*wt/wt (%)*	*wt/p.R4859K (%)*	*p.R4859K/ p.R4859K (%)[d]*
Members of 19 MMD families[a]				
Affected	42	0	39 (92.9)	3 (7.1)
Not affected	28	15 (53.6)	13 (46.4)	0
Individuals without a family history of MMD[b,c]				
Affected	63	17 (27.0)	45 (71.4)	1 (1.6)
Not affected	429	423 (98.6)	6 (1.4)	0

Abbreviations: MMD, moyamoya disease.
[a]Entire distribution, χ^2=29.4, P=4.2×10^{-7}.
[b]Entire distribution, χ^2=298.2, P=1.8×10^{-65}.
[c]Genotypic distribution (p.R4859K carrier vs non-carrier), χ^2=298.1, P=1.2×10^{-43}, odds ratio=190.8 (95% CI=71.7–507.9).
[d]The age of onset and initial symptoms of the four homozygotes were comparable to those of the 84 heterozygous patients.

in three patients heterozygous for the p.R4859K mutation (Figure 2b), excluding the possible instability of the mutant *RNF213* mRNA. Additional missense mutations, p.M3940V, p.V4616M and p.V4814M, were detected in three non-familial MMD cases without the p.R4859K mutation (Figure 2c). These mutations were not found in 388 control subjects and were detected in only one patient, suggesting that they were private mutations (Table 2). No copy number variation or mutation was identified in the *RNF213* locus of 12 MMD patients using comparative genome hybridization microarray analysis (Supplementary Figure 4). In total, 6 of the 429 control subjects (1.4%) were found to be heterozygous carriers of p.R4859K. Therefore, we concluded that the p.R4859K mutation increases the risk of MMD by a remarkably high amount (odds ratio=190.8 (95% confidence interval=71.7–507.9), P=1.2×10^{-43}) (Table 3). It was recently reported that an SNP (ss161110142) in the promoter region of *RPTOR*, which is located ~150 kb downstream from *RNF213*, was associated with MMD.[17] Genotyping of the SNP in *RPTOR* showed that the *RNF213* p.R4859K mutation was more strongly associated with MMD than ss161110142 (Supplementary Figure 1).

RNF213 encodes a protein with 5256 amino acids harboring a RING (really interesting new gene) finger motif, suggesting that it functions as an E3 ubiquitin ligase (Figure 2c). It also has an AAA ATPase domain, which is characteristic of energy-dependent unfoldases.[18] To our knowledge, RNF213 is the first RING finger protein known to contain an AAA ATPase domain. The expression profile of *RNF213* has not been previously fully characterized. We performed a quantitative reverse transcription PCR analysis in various human tissues and cells. *RNF213* mRNA was highly expressed in immune tissues, such as spleen and leukocytes (Figure 3a and Supplementary Figure 5). Expression of *RNF213* was detected in fractions of both polymorphonuclear cells and mononuclear cells and was found in both B and T cell fractions (Supplementary Figure 6). A low but significant expression of *RNF213* was also observed in human umbilical vein endothelial cells and human pulmonary artery smooth muscle cells. Cellular expression was not enhanced in tumor cell lines, compared with leukocytes. In human fetal tissues, the highest expression was observed in leukocytes and the thymus (Supplementary Figure 6E). The expression of *RNF213* was surprisingly low in both adult and fetal brains. Overall, *RNF213* was ubiquitously expressed, and the highest expression was observed in immune tissues.

We studied the cellular expression of *Rnf213* in mice. The ISH analysis of spleen showed that *Rnf213* mRNA was present in small mononuclear cells, which were mainly localized in the white pulps (Figures 3b–g). The ISH signals were also detected in the primary follicles in the lymph node and in thymocytes in the medulla of the thymus (Supplementary Figure 7). To study *Rnf213* expression in activated lymphocytes we immunized mice with keyhole limpet hemocyanin, and examined *Rnf213* mRNA in spleen by ISH analysis. Primary immunization with keyhole limpet hemocyanin antigen revealed that the expression of *Rnf213* in the secondary follicle is as high as in the primary follicle in the lymph node (Supplementary Figure 8). In an E16.5 mouse embryo, expression was observed in the medulla of the thymus and in the cells around the mucous palatine glands (Supplementary Figure 9). These findings suggest that mature lymphocytes in a static state express *Rnf213* mRNA at a higher level than do their immature counterparts.

DISCUSSION

We identified a susceptibility locus for MMD by genome-wide and locus-specific association studies. Further sequencing analysis revealed a founder missense mutation in *RNF213*, p.R4859K, which was tightly associated with MMD onset. Identification of a founder mutation in individuals with MMD would resolve the following recurrent

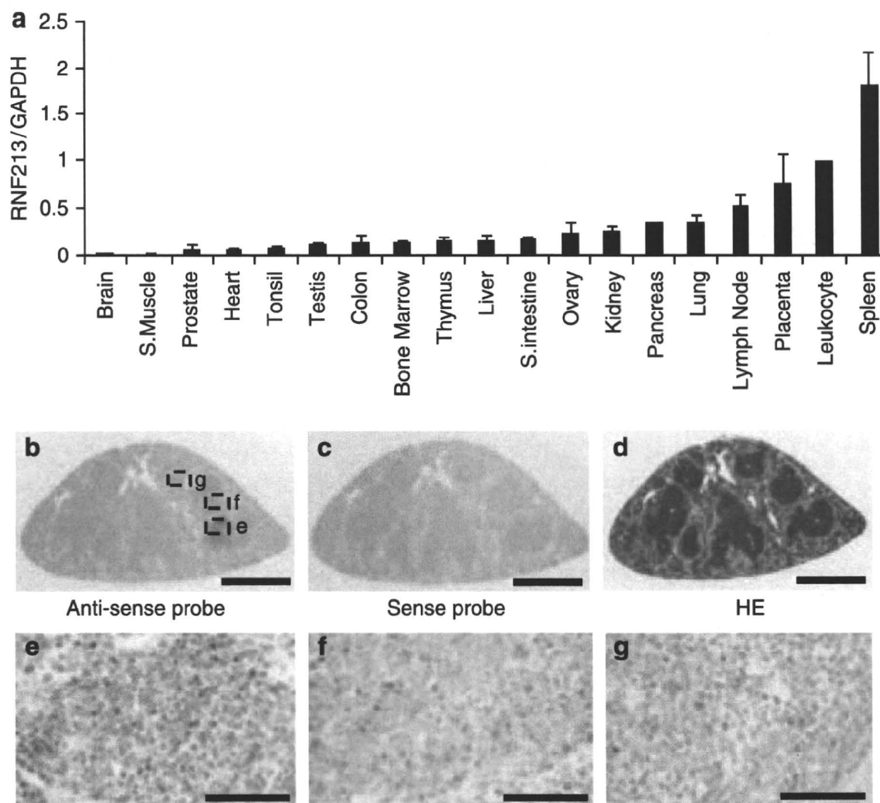

Figure 3 Expression of human RNF213 and murine Rnf213. (**a**) RT-PCR analysis of RNF213 mRNA in various human tissues. The expression levels of *RNF213* mRNA in various adult human tissues were evaluated by quantitative PCR using *GAPDH* mRNA as a control. The signal ratio of *RNF213* mRNA to *GAPDH* mRNA in each sample is shown on the vertical axis. (**b–g**) *In situ* hybridization (ISH) analysis of Rnf213 mRNA in mouse spleen. Specific signals for *Rnf213* mRNA were detected by ISH analysis with the anti-sense probe (**b**) but not with the sense probe (**c**). Hematoxylin–eosin staining of the mouse spleen (**d**). Signals for the *Rnf213* mRNA were observed in small mononuclear cells, which were mainly localized in the white pulps (dotted square, **e**) and partially distributed in the red pulps (dotted squares, **f** and **g**). Panels e, f and g show the high-magnification images of the corresponding fields in panel b. Scale bars, 1 mm (**b–d**) and 50 μm (**e–g**).

questions:[2,19] (i) why is MMD more prevalent in East Asia than in Western countries? The carrier frequency of p.R4859K in Japan is 1/72 (Table 2). In contrast, we found no p.R4859K carrier in 400 Caucasian controls (data not shown). Furthermore, no mutation was identified in five Caucasian patients with MMD after the full sequencing of *RNF213*. These results suggest that the genetic background of MMD in Asian populations is distinct from that in Western populations and that the low incidence of MMD in Western countries may be attributable to a lack of the founder *RNF213* mutation. (ii) Is unilateral involvement a subtype of MMD or a different disease?[2] We collected DNA samples from six patients with unilateral involvement and found a p.R4859K mutation in four of them (data not shown), suggesting that bilateral and unilateral MMD share a genetic background. (iii) Is pre-symptomatic diagnosis of MMD possible? In the present study, MMD never developed in the 15 mutation-negative family members in the 19 MMD families with the p.R4859K mutation (Table 3 and Supplementary Figure 1), suggesting the feasibility of presymptomatic diagnosis or exclusion by genetic testing.

How the mutant RNF213 protein causes MMD remains to be elucidated. The expression of *RNF213* was more abundant in a subset of leukocytes than in the brain, suggesting that blood cells have a function in the etiology of MMD. This observation agrees with a previous report that MMD patients have systemic angiopathy.[20] Recent studies have suggested that the postnatal vasculature can form through vasculogenesis, a process by which endothelial progenitor cell are recruited from the splenic pool and differentiate into mature endothelial cells.[21] Levels of endothelial progenitor cells in the peripheral blood are increased in MMD patients.[22] *RNF213* may be expressed in splenic endothelial progenitor cells and mutant *RNF213* might dysregulate the function of the endothelial progenitor cells. Further research is necessary to elucidate the role of *RNF213* in the etiology of MMD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Common Variants in the ATP2B1 Gene Are Associated With Susceptibility to Hypertension: The Japanese Millennium Genome Project

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Common Variants in the ATP2B1 Gene Are Associated With Susceptibility to Hypertension

The Japanese Millennium Genome Project

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Abstract—Hypertension is one of the most common complex genetic disorders. We have described previously 38 single nucleotide polymorphisms (SNPs) with suggestive association with hypertension in Japanese individuals. In this study we extend our previous findings by analyzing a large sample of Japanese individuals (n=14 105) for the most associated SNPs. We also conducted replication analyses in Japanese of susceptibility loci for hypertension identified recently from genome-wide association studies of European ancestries. Association analysis revealed significant association of the *ATP2B1* rs2070759 polymorphism with hypertension ($P=5.3\times10^{-5}$; allelic odds ratio: 1.17 [95% CI: 1.09 to 1.26]). Additional SNPs in *ATP2B1* were subsequently genotyped, and the most significant association was with rs11105378 (odds ratio: 1.31 [95% CI: 1.21 to 1.42]; $P=4.1\times10^{-11}$). Association of rs11105378 with hypertension was cross-validated by replication analysis with the Global Blood Pressure Genetics consortium data set (odds ratio: 1.13 [95% CI: 1.05 to 1.21]; $P=5.9\times10^{-4}$). Mean adjusted systolic blood pressure was highly significantly associated with the same SNP in a meta-analysis with individuals of European descent ($P=1.4\times10^{-18}$). *ATP2B1* mRNA expression levels in umbilical artery smooth muscle cells were found to be significantly different among rs11105378 genotypes. Seven SNPs discovered in published genome-wide association studies were also genotyped in the Japanese population. In the combined analysis with replicated 3 genes, *FGF5* rs1458038, *CYP17A1*, rs1004467, and *CSK* rs1378942, odds ratio of the highest risk group was 2.27 (95% CI: 1.65 to 3.12; $P=4.6\times10^{-7}$) compared with the lower risk group. In summary, this study confirmed common genetic variation in *ATP2B1,* as well as *FGF5*, *CYP17A1*, and *CSK*, to be associated with blood pressure levels and risk of hypertension. **(*Hypertension*. 2010;56:973-980.)**

Key Words: hypertension ■ genetic variation ■ ATP2B1 ■ Millennium Genome Project ■ Global BPgen

Because of its large impact on a number of cardiovascular diseases, hypertension is a major contributor to global health burden. Because hypertension is one of the most prevalent complex genetic disorders, with a heritability of ≤60% based on the estimation by 24-hour blood pressure (BP) readings,[1] numerous studies, including recent genome-wide association studies (GWAS),[2–6] have attempted to identify genetic variation associated with human BP levels.

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Except for rare mendelian forms of hypertension,[7] the estimated effects of each genetic factor on BP levels have been found to be small in the general population (typically <1.0 mm Hg on systolic BP [SBP] and <0.5 mm Hg on diastolic BP [DBP] per risk allele). However, multiple risk alleles are known to have a cumulative impact on several complex traits, including BP and hypertension risk.[3] In addition, it is anticipated that identification of novel susceptibility genes would lead to further understanding of disease pathogenesis.

As a part of a series of nationally based cooperative projects, the Millennium Genome Project (Millennium GPJ), we conducted multiple candidate gene analyses to identify susceptible genes and polymorphisms for hypertension. In a previously reported study,[6] we focused on 307 genes, which were genes encoding components of signal transduction pathways potentially related to BP regulation, including receptors, soluble carrier proteins, binding proteins, channels, enzymes, and G proteins. That study identified 38 single nucleotide polymorphisms (SNPs) as suggestively associated with hypertension by analysis of 758 hypertensive patients and 726 normotensive controls.[6] To extend our previous study, we have now genotyped all 38 of the SNPs in a replication panel composed of 1929 hypertensives and 1993 normotensives and have taken forward validated SNPs with further genotyping in a large Japanese genetic epidemiological cohort sample (n=14 105). An in silico validation analysis of our most promising loci was performed using the Global Blood Pressure Genetics (Global BPgen) consortium data set, a large-scale GWAS of samples of European descent.[2] Furthermore, we also conducted a replication analysis of recent European GWAS-derived susceptible loci for hypertension from Global BPgen[2] and CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) GWAS[3] in a Japanese large-scale general population sample (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).

Methods

Case and Control Subjects (Screening Panel)

Details of the screening panel subjects have been described previously.[6] Briefly, hypertensive patients and normotensive controls were recruited in the Asahikawa, Tokyo, Osaka, and Hiroshima regions of Japan according to the following criteria. Hypertensive subjects (n=758) had a previous diagnosis of hypertension at between 30 and 59 years of age and were either being treated with antihypertensive medication or had a SBP >160 mm Hg and/or DBP >100 mm Hg. They had a family history of hypertension in their parents and/or siblings and were not obese (body mass index [BMI] <25 kg/m^2). Normotensive controls (n=726) aged >45 years were recruited from the same regions. These individuals have never been treated with antihypertensive medications, and their SBP was <120 mm Hg and DBP <80 mm Hg. They had no family history of hypertension. All of the subjects were unrelated and were native Japanese.

Cohort-Based Population Samples

Seven independent study cohorts for cardiovascular diseases and related risk factors were combined to compose a large-scale Japanese genetic epidemiological population sample of 14 105. The Ohasama, Shigaraki, Takashima, Suita, and Nomura studies are general population-based genetic epidemiological studies. The study subjects were recruited via a medical checkup process for community residents. The 2 other cohorts, Yokohama and Matsuyama, are derived from employees of large manufacturing industries. The clinical parameters used in this study were obtained from personal health records during annual medical checkups. Further details of the study cohorts are described in the online Data Supplement.

Nested Case and Control Subjects Derived From the Cohort-Based Sample (Replication Panel)

Hypertensive cases and normotensive controls were chosen from the cohort-based population samples described above (n=11 569; the Suita study was excluded because of ethical issues). The selection criteria of the hypertensive and normotensive subjects were as follows: hypertensive subjects (n=1929) aged ≤64 years and either treatment with antihypertensive medication and/or SBP >160 mm Hg and/or DBP >90 mm Hg; normotensive subjects (n=1993) aged ≥40 years and having SBP <120 mm Hg and DBP <80 mm Hg; and no current use of antihypertensive medication and free from any history of cardiovascular disease.

Global BPgen (In Silico) Analyses

To investigate cross-validation of the most promising SNPs, we obtained results for 4 SNPs in the *ATP2B1* gene from the Global BPgen consortium, a study that is composed of 17 GWAS studies with 34 433 individuals of European descent. A detailed description of the study design and phenotype measurement for all of the cohorts has been reported previously.[2]

Validation of Published BP Polymorphisms in the Japanese Millennium Cohort

Thirteen loci have been identified recently and robustly validated for association with BP and hypertension in recent large-scale GWAS of European samples, by the Global BPgen consortium[2] and the CHARGE consortium.[3] From the associated SNPs reported at these 13 loci, we selected SNPs expected to have minor allele frequencies in Japanese samples >0.10, based on the HapMap database (JPT only, Public Release No. 27)[8]: *FGF5* rs1458038, *CYP17A1* rs1004467, *CSK* rs1378942, *PLCD3* rs12946454, *PLEKHA7* rs381815, *ULK4* rs9815354, and *CSK-ULK3* rs6495122. These 7 SNPs were genotyped in the Japanese population-based cohort sample to test whether the same associations exist in samples of Japanese ancestry.

Genotyping

Genomic DNA was extracted from peripheral blood. All of the SNPs were analyzed by TaqMan probe assays (Applied Biosystems Co, Ltd) using commercially available primers and probes purchased from the Assay-on-Demand system. The fluorescence level of PCR products was measured using an ABI PRISM 7900HT sequence detector.

Ethical Considerations

All of the study procedures were approved by the ethics committee of each university or research institute. Written informed consent was obtained from all of the participating subjects.

Ex Vivo Expression Analysis of ATP2B1 mRNA

Umbilical artery smooth muscle cells were isolated from umbilical cords obtained at delivery (n=34). Expression levels of ATP2B1 mRNA were analyzed by RT-PCR using a relative quantification method. Further details of the ex vivo expression analysis are described in the online Data Supplement.

Statistical Analysis

At each SNP, frequency differences in each genotype among hypertensive and normotensive subjects were assessed using a χ^2 test. Linkage disequilibrium (LD) coefficients were calculated using the Haploview software (Broad Institute).[9] Adjusted odds ratios for hypertension, as well as coefficients and SEs for SBP and DBP, were calculated using logistic and linear multiple regression analysis,

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Table 1. Association of ATP2B1 SNPs With Hypertension in the Screening and Replication Panels

			Screening Panel						Replication Panel						
SNP	Genotype		Genotype Frequency			HWE	Call Rate	Odds (*P*)	Genotype Frequency			HWE	Call Rate	Odds (*P*)	Overall Odds (*P*)
rs1401982	AA/AG/GG	HT	318	328	92	0.603	96.3	1.28 (0.001)	825	833	247	0.108	98.7	1.25 (3.0×10^{-6})	1.26 (1.5×10^{-8})
		NT	249	324	118	0.474			699	961	305	0.397			
rs2681472	AA/AG/GG	HT	335	321	90	0.334	97.8	1.26 (0.003)	846	832	242	0.095	99.5	1.26 (1.0×10^{-6})	1.26 (8.7×10^{-9})
		NT	267	328	111	0.539			715	966	303	0.431			
rs2070759	GG/GT/TT	HT	216	379	151	0.515	97.6	1.16 (0.045)	582	896	399	0.118	97.2	1.18 (4.4×10^{-4})	1.17 (5.3×10^{-5})
		NT	186	341	175	0.454			507	956	474	0.579			
rs11105364	TT/TG/GG	HT	335	322	88	0.432	97.2	1.29 (0.001)	846	834	236	0.171	99.3	1.25 (2.4×10^{-6})	1.26 (4.1×10^{-9})
		NT	261	323	113	0.438			729	947	303	0.874			
rs11105378	CC/CT/TT	HT	359	301	76	0.276	97.3	1.37 (6.3×10^{-5})	868	821	217	0.280	98.8	1.28 (1.4×10^{-7})	1.31 (4.1×10^{-11})
		NT	280	320	108	0.295			746	922	300	0.586			

The screening panel is composed of 758 middle age–onset severe hypertensive patients and 726 middle-aged to elderly evidently normotensive controls (Table S4). The replication panel consists of 1929 hypertensive cases, and 1993 normotensive controls selected from 11 569 cohort sample were enrolled (Table S2). ORs and *P* values for allelic model are shown.

adjusting for sex, age, age^2, BMI, and cohort variables, using additive (1 degree of freedom) and genotypic (2 degrees of freedom) genetic models. Adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15 mm Hg for SBP and +10 mm Hg for DBP).[10] The Global BPgen data and statistical methods have been described elsewhere.[2] Meta-analysis was performed assuming fixed effects and using inverse variance weights. An unweighted genetic risk score based on 4 SNPs (*ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1* rs1004467, and *CSK* rs1378942) was calculated by adding the number of risk alleles showing higher BP values. Risk allele of each SNP was defined as follows: *ATP2B1*, C allele; *FGF5*, T allele; *CYP17A1*, A allele; and *CSK*, C allele. The *CSK-ULK3* SNP rs6495122 showing positive association with BP trait and hypertension was not included in the calculation of genetic risk score, because the strong LD with the *CSK* SNP rs1378942 ($D'=0.884$; $r^2=0.731$) is most parsimoniously explained by both SNPs tagging a single risk variant. Differences in mRNA expression levels among the *ATP2B1* rs1105378 genotype were assessed by ANOVA. The statistical analyses were performed using a commercially available statistical software package (JMP version 8, SAS Institute).

Results

Replication Genotyping

The clinical characteristics of the replication panel chosen from the cohort-based population samples (Table S1, available in the online Data Supplement) are shown in Table S2. Stringent case and control definitions, corresponding with the extreme upper ≈17% and lower ≈17% of the general population, were used to maximize power for fixed genotyping costs.[11] Thirty-six SNPs were successfully genotyped, and results for all of the SNPs are shown in Table S3. Significant association was observed for the *ATP2B1* rs2070759 polymorphism located in intron 8 ($P=4.4\times10^{-4}$; allele odds ratio [OR]: 1.18 [95% CI: 1.07 to 1.29]). Several other SNPs also showed marginally significant association; however, the *P* values did not reach statistical significance after application of Bonferroni correction for multiple comparisons (threshold: 0.05/36=0.0014; Table S3; we note that no other SNPs are significant if the less conservative Benjamini-Hochberg procedure is used to control the false discovery rate at 0.05). Although, the replication results in the less-strict nested case-control sample chosen from the same population sample have been reported in our previous article,[6] the association was recalculated to narrow down the SNPs to be applied to the following dense SNP analysis.

Dense SNP Analysis of the ATP2B1 Gene

To more precisely identify the SNP or SNPs increasing susceptibility for hypertension, we performed "de novo" genotyping of a dense SNP panel around marker rs2070759 in individuals from the original screening panel (Table S4).[6] Forty-one tag SNPs located in a 167-kb region around rs2070759 were selected using the HapMap database (Table S5).[8] Among the 27 SNPs polymorphic in our Japanese sample, the most significant association was observed with rs11105378; this yielded an allelic *P* value of 6.3×10^{-5} (OR: 1.37 [95% CI: 1.17 to 1.60]; Table 1 and Figure S2).

The most associated SNP and the 4 others from the dense SNP analyses were subsequently genotyped in the replication panel. Significant association of rs11105378 was confirmed in the replication panel with an allelic *P* value of 1.4×10^{-7} (OR: 1.28 [95% CI: 1.17 to 1.41]; Table 1). Meta-analysis of both study panels indicated significant association ($P=4.1\times10^{-11}$; OR: 1.31 [95% CI: 1.21 to 1.42]) and confirmed that the strongest association is seen for rs11105378. The D′ and r^2 measures of LD between rs2070759 and rs11105378 were 0.92 and 0.48, respectively. Other SNPs, rs1401982 ($D'=0.99$; $r^2=0.64$), rs2681472 ($D'=0.99$; $r^2=0.61$), rs11105364 ($D'=0.97$; $r^2=0.59$), located within the same LD block, were also significantly associated with hypertension (Table 1). The strong LD between associated SNPs suggests a single true association signal in this region.

We examined for possible association of SNPs in the *ATP2B4* gene, a well-investigated isoform of the *ATP2B1* gene, with hypertension in the screening panel. We observed no significant correlation with the 17 SNPs analyzed, which were selected using the HapMap database (Table S6).

Population-Based Meta-Analyses of ATP2B1 SNPs

The complete Japanese population-based sample was subsequently genotyped for the 4 most significant SNPs in

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Table 2. Meta-Analysis of ATP2B1 SNPs With BP Traits

		Millennium GPJ			Global BPgen			CHARGE*			Pooled	
SNP	Coded Allele	n (Frequency)	Coefficient (SE), mm Hg	P	n (Frequency)	Coefficient (SE), mm Hg	P	n (Frequency)	Coefficient (SE), mm Hg	P	Coefficient (95% CI), mm Hg	P
SBP												
rs1401982	G	13 944 (0.376)	−1.22 (0.23)	1.8×10^{-7}	33 885 (0.385)	−0.30 (0.13)	0.022				−0.52 (−0.74 to −0.30)	3.9×10^{-6}
rs2681472	G	14 032 (0.373)	−1.33 (0.23)	1.2×10^{-8}	33 803 (0.158)	−0.62 (0.18)	5.2×10^{-4}	0.17	−1.29 (0.19)	3.5×10^{-11}	−1.03 (−1.26 to −0.81)	9.9×10^{-20}
rs11105364	G	14 013 (0.364)	−1.34 (0.23)	8.9×10^{-9}	33 877 (0.179)	−0.60 (0.18)	7.4×10^{-4}	0.17	−1.30 (0.19)	4.8×10^{-11}	−1.03 (−1.25 to −0.81)	1.2×10^{-19}
rs11105378	T	13 948 (0.360)	−1.33 (0.23)	1.5×10^{-8}	33 171 (0.158)	−0.59 (0.18)	0.001	0.16	−1.31 (0.20)	9.1×10^{-11}	−1.02 (−1.24 to −0.79)	1.4×10^{-18}
DBP												
rs1401982	G	13 944 (0.376)	−0.72 (0.14)	2.0×10^{-7}	33 898 (0.392)	−0.18 (0.09)	0.041				−0.34 (−0.49 to −0.19)	8.1×10^{-6}
rs2681472	G	14 032 (0.373)	−0.65 (0.14)	2.7×10^{-6}	33 829 (0.157)	−0.35 (0.12)	0.003	0.17	−0.64 (0.11)	3.7×10^{-8}	−0.54 (−0.68 to −0.41)	9.7×10^{-15}
rs11105364	G	14 013 (0.364)	−0.70 (0.14)	4.5×10^{-7}	33 898 (0.158)	−0.34 (0.12)	0.004	0.17	−0.63 (0.12)	1.2×10^{-7}	−0.54 (−0.68 to −0.40)	7.5×10^{-14}
rs11105378	T	13 948 (0.360)	−0.70 (0.14)	5.4×10^{-7}	33 183 (0.158)	−0.33 (0.12)	0.005	0.16	−0.62 (0.12)	3.1×10^{-7}	−0.54 (−0.68 to −0.39)	1.6×10^{-13}

Coefficients and SE for SBP and DBP were calculated under the additive model using multiple regression analysis adjusted for age, age^2, sex, and BMI. In both Millennium GPJ and Global BPgen, adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15 mm Hg for SBP and +10 mm Hg for DBP).[2] In the Japanese Millennium GPJ and also for some cohorts within Global BPgen, cohort variables were also adjusted to avoid residual population stratification.

*Results of the CHARGE Study were obtained from the published article.[3]

ATP2B1. To further validate and get more precise effect size estimates in Japanese, for this analysis, hypertensive cases were defined as individuals with treatment with antihypertensive medication, SBP ≥140 mm Hg, or DBP ≥90 mm Hg. The ORs for the 4 SNPs were all extremely similar (ranging from 1.19 to 1.21 under the additive model adjusted for age, age^2, sex, BMI, and cohort variables; see Table S7). These associations were replicated in the Global BPgen subjects of European descent; the pooled analysis demonstrated increased significance (rs1105378: OR: 1.17 [95% CI: 1.11 to 1.23]; $P=7.0\times10^{-10}$), as expected for a larger total sample size (n=28 866; Table S7).

We next evaluated the effect of the most associated SNP, rs11105378, on BP levels in the Millennium GPJ cohort (Table 2). We adjusted for several covariates that are associated with BP phenotypes: age ($r=0.362$; $P<0.001$ for SBP), BMI ($r=0.275$; $P<0.001$), and sex (male: 131.7±18.2; female: 128.6±20.8 mm Hg; $P<0.001$). In multiple regression analysis for BP levels, including also cohort indicator variables as covariates, the results for a 2-degree-of-freedom test with the TT genotype as a reference identified both the TC genotype (coefficient=+1.66 mm Hg; $P=2.2\times10^{-4}$) and CC genotype (+2.47 mm Hg; $P=4.9\times10^{-8}$) as independent determinants for SBP after adjustment. The TC (+0.91 mm Hg; $P=8.0\times10^{-4}$) and CC genotypes (+1.32 mm Hg; $P=1.8\times10^{-6}$) were also independently associated with DBP levels. We depict the covariate adjusted mean BP levels by rs11105378 genotype in Figure S3. Results of each cohort separately are summarized in Table S8. We next performed a meta-analysis of data from the Millennium GPJ and 2 large epidemiological studies (Global BPgen and CHARGE; Table 2). Results show the per-allele differences in SBP and DBP to be ≈1.0 and 0.5 mm Hg, respectively.

Genotype-Specific Differences in Ex Vivo Expression of ATP2B1 mRNA

Differences in *ATP2B1* mRNA expression in umbilical artery smooth muscle cells among rs11105738 genotype are shown in Figure 1. Assuming a recessive genetic model, cells homozygous for T allele showed significantly higher levels of

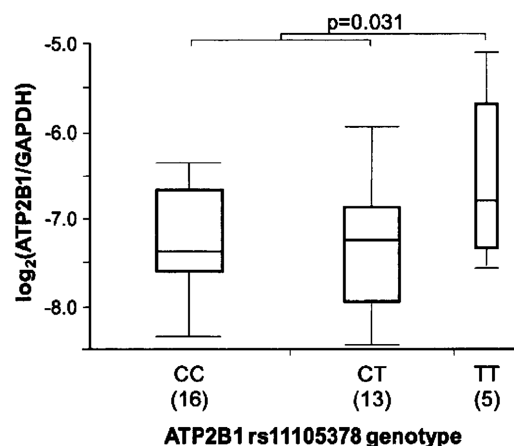

Figure 1. Ex vivo expression analysis of *ATP2B1* mRNA. Graphs depict the log^2 relative expression levels of the *ATP2B1* mRNA in umbilical artery smooth muscle cells obtained by normalizing to GAPDH. Genotype of *ATP2B1* rs11105378 of each sample was analyzed by direct sequencing using isolated genomic DNA from umbilical artery smooth muscle cells.

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Table 3. Meta-Analysis of SNPs With BP Traits

		Millennium GPJ			Global BPgen			Pooled	
SNP	Coded Allele	n (Frequency)	Coefficient (SE), mm Hg	*P*	n (Frequency)	Coefficient (SE), mm Hg	*P*	Coefficient (95% CI), mm Hg	*P*
Systolic BP									
FGF5	T	13 826	1.33	1.6×10^{-8}	30 850	0.62	1.6×10^{-6}	0.81	1.1×10^{-11}
rs1458038		(0.343)	(0.23)		(0.275)	(0.14)		(0.58 to 1.05)	
CYP17A1	A	14 007	0.89	2.3×10^{-4}	33 735	0.94	1.0×10^{-5}	0.92	6.2×10^{-9}
rs1004467		(0.680)	(0.24)		(0.901)	(0.21)		(0.61 to 1.23)	
CSK	C	13 920	0.77	0.007	34 126	0.62	2.4×10^{-6}	0.65	4.2×10^{-8}
rs1378942		(0.803)	(0.28)		(0.36)	(0.13)		(0.42 to 0.88)	
PLCD3	T	14 003	0.11	0.703	32 120	0.68	3.9×10^{-6}	0.57	2.5×10^{-5}
rs12946454		(0.831)	(0.30)		(0.28)	(0.15)		(0.30 to 0.83)	
PLEKHA7	T	14 030	0.11	0.687	33 706	0.52	2.6×10^{-4}	0.44	4.7×10^{-4}
rs381815		(0.199)	(0.28)		(0.26)	(0.14)		(0.19 to 0.68)	
CSK-ULK3	A	14 014	0.68	0.017	33 308	0.47	2.4×10^{-4}	0.51	1.7×10^{-5}
rs6495122		(0.812)	(0.28)		(0.45)	(0.13)		(0.28 to 0.74)	
ULK4	A	13 976	−0.67	0.059	32 034	0.17	0.297	0.01	0.950
rs9815354		(0.116)	(0.35)		(0.18)	(0.17)		(−0.29 to 0.31)	
DBP									
FGF5	T	13 826	0.73	1.8×10^{-7}	30 850	0.55	1.5×10^{-8}	0.61	6.1×10^{-14}
rs1458038		(0.343)	(0.14)		(0.275)	(0.10)		(0.45 to 0.77)	
CYP17A1	A	14 007	0.29	0.047	33 735	0.40	5.4×10^{-3}	0.35	4.9×10^{-4}
rs1004467		(0.680)	(0.14)		(0.901)	(0.14)		(0.15 to 0.54)	
CSK	C	13 920	0.41	0.015	34 126	0.48	5.9×10^{-8}	0.46	5.2×10^{-9}
rs1378942		(0.803)	(0.17)		(0.36)	(0.09)		(0.31 to 0.62)	
PLCD3	T	14 003	0.14	0.426	32 120	0.34	5.7×10^{-4}	0.30	1.9×10^{-4}
rs12946454		(0.831)	(0.18)		(0.28)	(0.09)		(0.14 to 0.46)	
PLEKHA7	T	14 030	0.13	0.437	33 706	0.23	0.014	0.20	0.018
rs381815		(0.199)	(0.17)		(0.26)	(0.10)		(0.04 to 0.37)	
CSK-ULK3	A	14 014	0.38	0.027	33 308	0.35	4.2×10^{-5}	0.36	7.4×10^{-6}
rs6495122		(0.812)	(0.17)		(0.45)	(0.09)		(0.20 to 0.51)	
ULK4	A	13 976	0.21	0.325	32 034	0.40	2.9×10^{-4}	0.36	2.3×10^{-4}
rs9815354		(0.116)	(0.21)		(0.18)	(0.11)		(0.17 to 0.55)	

ATP2B1 mRNA as compared with cells carrying 1 or 2 C alleles ($P=0.031$; see Figure 1). Under an additive genetic model, the overall *P* value was marginally significant ($P=0.091$).

Replication Analysis of European GWAS-Derived Susceptible SNPs in Japanese

We next conducted a replication analysis in the Millennium GPJ, in which we tested associated SNPs identified in recent large-scale European GWAS by the Global BPgen[2] and the CHARGE consortia.[3] From the 7 most promising SNPs of which the minor allele frequency in Japanese was >0.10 based on the HapMap database, 4 SNPs, namely, *FGF5* rs1458038, *CYP17A1* rs1004467, *CSK* rs1378942, and *CSK-ULK3* rs6495122, showed significant association in either binary trait analyses (Tables S9) or quantitative trait analysis (Table 3 and S10). The most significant association was observed with *FGF5* rs1458038; this yielded a *P* value of 1.6×10^{-8} (+1.33 mm Hg) with SBP and 1.8×10^{-7} (+0.73 mm Hg) with DBP in the Millennium GPJ cohort, and the effect size was greater than that of Europeans (Table 3). Meta-analysis of both study panels with data from Global BPgen indicated further significant associations.

Multiple Regression Analysis for BP Trait and Hypertension in Japanese

To clarify whether the 4 susceptibility SNPs (*ATP2B1*, *FGF5*, *CYP17A1*, and *CSK*) were independently associated with BP traits and hypertension, multiple regression analysis was performed with possible covariates (Table S11). After adjustment for age, age^2, sex, BMI, and drinking habits, this analysis confirmed that all 4 of the SNPs were independent determinants for both BP traits and hypertension.

Combined Effect of Risk Genotypes on Hypertension

A risk score for 4 susceptible genotypes was calculated to evaluate their combined effects on hypertension. ORs asso-

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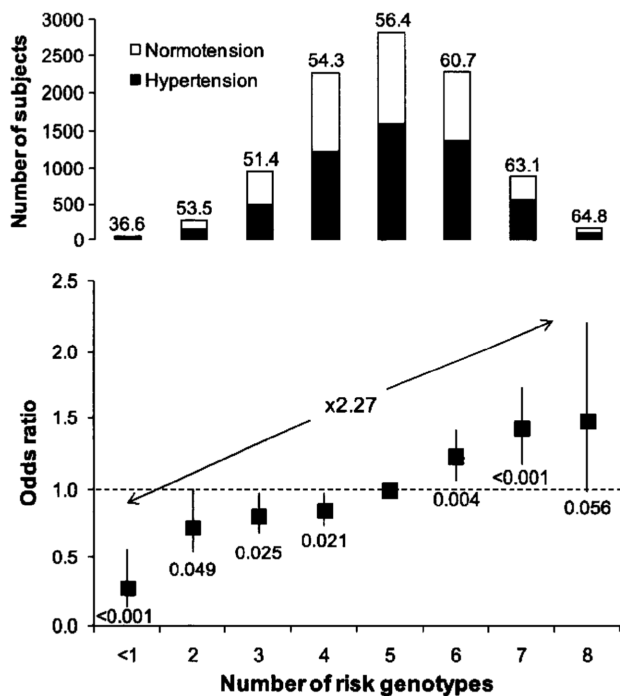

Figure 2. ORs for hypertension according to the number of risk genotypes Number of risk genotype was calculated by the following 4 SNPs: *ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1*, rs1004467, and *CSK* rs1378942. Hypertensive subjects were defined as being treated with antihypertensive medication, SBP ≥140 mm Hg, or DBP ≥90 mm Hg; normotensive subjects were defined as all not treated with antihypertensive medication, SBP ≤120 mm Hg, and DBP ≤85 mm Hg.[2] Adjusted OR for hypertension and BP levels were calculated using logistic and linear multiple regression analysis, adjusting for sex, age, age^2, BMI, and cohort variables. Frequency of hypertension and *P* values for the hypertension odds are shown in the top of column and the bottom of square, respectively.

ciated with increasing number of risk genotypes in a covariates adjusted logistic regression model are depicted in Figure 2 (overall *P* value was 5.4×10^{-5}). Compared with the reference group (5 risk genotypes), individuals carrying 7 or 8 risk genotypes had higher risk (OR: 1.43 [95% CI: 1.20 to 1.72]; $P=1.0\times10^{-4}$) in contrast to the lower OR of individuals with ≤2 risk genotypes (OR: 0.63 [95% CI: 0.47 to 0.85]; $P=0.020$). The OR of the high-risk group was raised to 2.27 (95% CI: 1.65 to 3.12; $P=4.6\times10^{-7}$) compared with the lowest risk group. Adjusted per-allele OR for hypertension was 1.17 (95% CI: 1.12 to 1.21; $P=4.0\times10^{-15}$). The distribution of the Japanese population sample among the number of risk genotypes is shown in Figure S4.

Discussion

The present study has identified SNPs located upstream or within the *ATP2B1* gene as strong susceptibility polymorphisms for hypertension in Japanese. These are findings that have also been reported recently in individuals of European descent[3] and in Koreans.[4] Although numerous studies have attempted to identify genetic markers for hypertension over the past 2 decades, there has been little cross-validation of loci in different ethnic groups so far except for mendelian forms of hypertension. The SNPs in *ATP2B1* identified in this study showed significant association in large-scale studies in populations with different ancestries and using different discovery approaches, including GWAS in the CHARGE consortium and the Korean study and an independent candidate gene analysis in our present study. Similar findings in different ethnic groups with different methods further strengthen these findings and indicate the *ATP2B1* gene region as a susceptibility locus of likely global significance for BP variation and development of hypertension. Two replication results very recently reported by another Japanese group[12] and a Korean group[13] also indicated the disease susceptibility of *ATP2B1* SNPs located in the same LD block.

No biological data have been provided whether SNP rs1105378 or other SNPs in strong LD have any effect on the transcriptional activity or transcriptional regulation of the *ATP2B1* gene. Furthermore, although alternative splicing has been found to generate several variants of *ATP2B1* mRNA,[14] the SNP associations that we have observed do not shed light on whether this is a potential mechanism for affecting BP. Our data first showed that the effect of SNPs on ATP2B1 gene expression levels is a potential mechanism by which disease-associated SNP alleles cause the phenotypic changes. Changes in the *ATP2B1* gene product levels are involved in BP regulation. We found no microRNA harboring rs11105378 in the miRBase database.[15]

The *ATP2B1* (so-called *PMAC1*) gene encodes the plasma membrane calcium ATPase isoform 1, which removes bivalent calcium ions from eukaryotic cells against very large concentration gradients and plays a critical role in intracellular calcium homeostasis. Although pathophysiological implications of *ATP2B1* gene products on the development of hypertension are uncertain, it has been reported that inhibition of *ATP2B1* by the selective inhibitor caloxin 2A1 showed endothelium-dependent relaxation of rat aorta by increasing cytosolic Ca^{2+} concentration and consequent activation of endothelial NO synthase.[16] Other information on the role of *ATP2B1* has been obtained from experiments using bladder smooth muscle cells: contractility measurements on these cells have documented the important role of *ATP2B1* in the extrusion of Ca^{2+} after carbachol stimulation or depolarization with potassium chloride.[17] These reports suggest altered vascular reactivity as a plausible explanation for disease susceptibility of *ATP2B1* gene.

In mammals, calcium ATPase isoforms are encoded by ≥4 separate genes (*ATP2B1* to *ATP2B4*).[18] It has been reported that overexpression of the human *ATP2B4* gene in arterial smooth muscle cells in mice increases vascular reactivity and BP partly because of negative regulation of neuronal NO synthase.[19] We, therefore, examined the possible association of *ATP2B4* gene polymorphisms with hypertension by using the screening panel. However, no significant correlation was observed in the 17 SNPs analyzed, which were selected by reference to the HapMap database. The pathophysiological association of plasma membrane Ca^{2+} pump with BP regulation may be isoform specific.

Numerous studies, including the recent GWAS,[3–6] have attempted to identify genetic variations associated with human BP levels. At present, it is not clear to what extent findings from GWAS in one population can be extrapolated

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to other populations with different lifestyles and genetic background. However, the present study provides a cross-validation of 4 of 7 SNPs (most likely representing 3 of 6 independent signals) derived from European GWAS. Replication studies in other Japanese[12] and Korean[13] populations also reported the cross-validation of European GWAS-derived SNP. Conservation of susceptible loci for hypertension was independent of ethnic background. This finding suggests an existence of unidentified common etiology of essential hypertension in relation to the susceptible genes and their physiological pathways.

Although individual common genetic variants confer a modest risk of hypertension, their combination showed a large impact on hypertension. The genetic risk score was associated with ≤2.27-times greater odds for hypertension. Similar observations have been found in other common diseases and multifactorial phenotypes, including, for example, type 2 diabetes mellitus,[20] serum lipid levels,[21] and serum uric acid levels.[22] We reported previously that the findings of the cross-sectional analysis revealed a similar association in the longitudinal analysis[23]; the fat mass and obesity-associated gene polymorphism was an independent risk factor for the future development of obesity after adjustment for possible confounding factors. The present cross-sectional study cannot address the question of whether the *ATP2B1* polymorphism and other susceptible variants predict future development of hypertension. However, recent articles investigating a prognostic significance of susceptible variants for type 2 diabetes mellitus[24] and cardiovascular disease[25] showed poor predictive performance of common variants in spite of the high OR observed in subjects carrying multiple risk alleles. A small proportion of the genetically high-risk persons attributed to independent inheritance of risk alleles may make it difficult to discriminate intermediate-risk persons. Genetic information may be most useful to identify a high-risk individual's need for early intervention.

Several definitions of hypertension were used in this study to explore susceptible SNPs with modest effects and to further validate the susceptibility. Since it was expected to be underpowered to detect the effects of common variants in a dichotomized analysis with slightly elevated BP, subjects with high normal BP were excluded from the 65 347 case-control analyses. All of the alleles associated with hypertension in a dichotomized analysis (Table S7) were also associated with BP levels (Table 2). Our methodology may, thus, be appropriate to identify susceptible variants for hypertension.

Perspectives

We have identified SNPs located in the *ATP2B1* gene region as susceptibility loci for hypertension in Japanese using a multistage association study, an association that has now been confirmed across different ethnic groups. Differences in the ex vivo *ATP2B1* mRNA expression levels further supported the disease susceptibility of SNP rs1110578. We also replicated the susceptibility of the European GWAS-derived SNPs in Japanese. Because hypertension is a trait that is preventable by dietary and exercise interventions, early detection of at-risk populations using genetic information may be useful in preventing future hypertension-related diseases.

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Disclosures

Several authors (Y.T., K.K., Y.Ki., N.H., J.N., S.U., H.U., and T.Mik.) have been named as inventors on a patent application by Ehime University, Shiga University of Medical Science, and Yokohama City University in work related to this study.

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ONLINE SUPPLEMENT

Common variants in the ATP2B1 gene are associated with susceptibility to hypertension
The Japanese Millennium Genome Project

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SUPPLEMENTAL METHODS

ex vivo expression analysis of ATP2B1 mRNA

We obtained 34 umbilical cords at delivery (Kosei General Hospital). Umbilical arteries were excised from the cords and cut into small pieces. Umbilical artery smooth muscle cells (UASMCs) were separated using Hanks buffer containing 2 mg/ml collagenase and cultured in HuMedia-SG (Kurabo, Osaka, Japan) supplemented with epithelial growth factor (0.5 ng/ml), basic fibroblast growth factor (2 ng/ml), insulin (5 μg/ml), antibiotics and 5% fetal bovine serum. Total RNAs was extracted from UASMCs during early passages using TRIzol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 500 ng of the total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), and then diluted five times for subsequent real-time PCR (RT-PCR). RT-PCR was performed using TaqMan Gene Expression Assays on a 7900HT Sequence Detection System (Applied Biosystems). A relative quantification method [1] was used to measure the amounts of ATP2B1 (TaqMan assay ID, Hs00155949_m1) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) as an internal control. Genotype of ATP2B1 rs11105378 of each sample was analyzed by direct sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit on a 3730xl GeneticAnalyzer, Applied Biosystems) using isolated genomic DNA from UASMCs (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany). The direct sequencing was performed with the following primers; forward 5'-TTCATAGCCCTTTTCATCTCTTTC-3', reverse 5'-AGAATCTCGGGAAAACAGCA-3'.

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Table S1 Clinical characteristics of the cohort-based population sample

Parameters	Total (14,105)	Community-based general population					Company employee	
		Ohasama (1,592)	Shigaraki (2,273)	Takashima (1,730)	Suita (2,536)	Nomura (2,876)	Yokohama (2,290)	Matsuyama (808)
Age (years)	57.8±14.0	57.5±11.2	57.2±15.5	59.7±14.1	65.6±10.9	61.1±14.0	45.7±10.2	54.2±5.8
Sex (male/female)	6931/7174	601/991	862/1411	633/1097	1160/1376	1247/1629	1659/631	769/39
Body mass index (kg/m^2)	23.0±3.1	23.7±3.2	22.6±3.1	22.9±3.0	22.9±3.1	23.4±3.2	22.4±3.1	23.4±2.9
History of CVD	7.1	11.9	12.1	4.0	7.5	8.1	0.4	4.3
Systolic BP (mmHg)	130.1±19.6	131.7±14.2	130.1±19.5	130.6±21.3	124.5±18.9	137.7±22.1	123.8±14.9	134.3±19.1
Diastolic BP (mmHg)	77.9±11.5	74.4±9.4	76.7±11.7	76.8±12.0	75.6±10.5	81.0±11.8	78.3±10.3	85.1±12.2
Hypertension (%)	40.7	43.2	44.4	39.5	38.2	53.3	22.9	46.2
Antihypertensive treatment (%)	20.5	26.5	23.5	16.4	26.4	25.7	6.5	12.4

Values are mean±SD. Cardiovascular disease (CVD); stroke, myocardial infarction, and angina pectoris. Hypertension; any or all of systolic blood pressure more than 140 mmHg, diastolic blood pressure more than 90 mmHg, and current use of antihypertensive agents. The Ohasama study conducted by Tohoku University is a population-based longitudinal epidemiological study focusing on the clinical implications of home BP measurement [2]. Ohasama Town is a rural community located in the northern part of Japan (Iwate Prefecture). Subjects were recruited through a community-based annual medical check-up process. The Shigaraki [3] and Takashima [4]| studies of Shiga University of Medical Science are general population-based longitudinal studies. Both towns are located in central Japan (Shiga Prefecture). Subjects were recruited through a community-based annual medical check-up process. The Suita study conducted by the National Cardiovascular Center is based on the residents of Suita city, an urban city located in the second largest area Osaka, Japan [5]. Subjects were recruited through a biennial medical check-up process of the National Cardiovascular Center. The Nomura study of Ehime University is a longitudinal epidemiological study based on the Nomura Town residents, a largely rural community located in Ehime Prefecture [6]. Subjects were recruited through a community-based annual medical check-up process. The Yokohama (Yokohama City University) and Matsuyama (Ehime University) cohorts are derived from employees of large manufacturing industries located in Kanagawa and Matuyama City, Ehime Prefecture (western part of Japan) [7] respectively. In all cohorts, clinical parameters were obtained from personal health records during the annual or biennial medical check-up process. All study procedures were approved by the ethics committee of each University or Institution. Singed informed consent was obtained from all participating subjects.

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Table S2 Clinical characteristics of the replication panel

Parameters	Hypertensive cases (1,929)	Normotensive controls (1,993)	p
Age (years)	55.1±7.1	55.2±9.5	0.680
Sex (male/female)	1,200/729	829/1,164	<0.001
Body mass index (kg/m^2)	24.4±3.1	21.9±2.7	<0.001
History of CVD (%)	5.4	0	<0.001
Systolic blood pressure (mmHg)	146.3±15.9	109.5±7.5	<0.001
Diastolic blood pressure (mmHg)	91.0±10.1	67.7±6.5	<0.001
Antihypertensive treatment (%)	47.5	0	<0.001

Values are mean±SD. Nested hypertensive cases and normotensive control subjects were chosen from the cohort-based population sample according to the following criteria: hypertensive subjects aged 64 years or younger, and were either being treated with antihypertensive medication or had a SBP more than 160 mmHg and/or DBP more than 90 mmHg; normotensive subjects aged 40 years or older, and all of SBP less than 120 mmHg, and DBP less than 80 mmHg, no current use of antihypertensive medication, and free from any history of cardiovascular disease. Cardiovascular disease (CVD) includes stroke, myocardial infarction, and angina pectoris.

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Table S3 Association of 36 candidate SNPs with hypertension (replication panel)

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ACCN1	rs28933	AA/GA/GG	HT	464	974	449	0.159	97.6	1.03 (0.479)	1.07 (0.385)	1.02 (0.766)	(0.686)
			NT	469	986	485	0.466					
ADORA1	rs3766554	AA/GA/GG	HT	424	923	557	0.262	98.6	1.03 (0.548)	1.00 (0.977)	1.09 (0.289)	(0.523)
			NT	410	981	574	0.808					
ATP10A	rs3736186	GG/AG/AA	HT	791	868	263	0.312	99.4	**1.10 (0.040)**	1.04 (0.666)	**1.18 (0.010)**	**(0.033)**
			NT	734	963	280	0.206					
ATP10D	rs1058793	AA/GA/GG	HT	675	894	325	0.326	98.2	1.07 (0.147)	1.17 (0.060)	1.04 (0.555)	(0.169)
			NT	680	896	382	0.005					
ATP2A3	rs887387	TT/TC/CC	HT	936	775	189	0.126	98.7	1.05 (0.342)	1.02 (0.840)	1.07 (0.263)	(0.527)
			NT	936	836	200	0.508					
ATP2B1	rs2070759	GG/GT/TT	HT	582	896	399	0.118	97.2	**1.18 ($4.0*10^{-4}$)**	**1.2 (0.018)**	**1.27 (0.001)**	**(0.002)**
			NT	507	956	474	0.579					
CACNA1E	rs2293990	AA/TA/TT	HT	568	911	412	0.194	98.2	1.03 (0.532)	1.07 (0.372)	1.01 (0.881)	(0.661)
			NT	585	926	451	0.022					
CACNA2D2	rs2236957	GG/GA/AA	HT	459	925	496	0.499	97.3	1.00 (0.948)	1.00 (0.972)	1.01 (0.943)	(0.997)
			NT	471	954	512	0.523					
CAST	rs967591	AA/AG/GG	HT	442	916	552	0.100	99.1	1.00 (0.932)	0.98 (0.725)	1.02 (0.814)	(0.875)
			NT	451	964	561	0.345					
CHGA	rs3759717	CC/TC/TT	HT	744	877	288	0.263	99.1	1.00 (0.977)	0.93 (0.434)	1.04 (0.598)	(0.522)
			NT	755	943	281	0.624					
COL4A1	rs2305080	GG/GA/AA	HT	485	908	523	0.023	99.2	1.02 (0.723)	0.97 (0.707)	1.07 (0.332)	(0.468)
			NT	473	972	528	0.536					

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