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REVIEW SERIES

Hunting for genes for hypertension: the Millennium Genome Project for Hypertension

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The Millennium Genome Project for Hypertension was started in 2000 to identify genetic variants conferring susceptibility to hypertension, with the aim of furthering the understanding of the pathogenesis of this condition and realizing genome-based personalized medical care. Two different approaches were launched, genome-wide association analysis using single-nucleotide polymorphisms (SNPs) and microsatellite markers, and systematic candidate gene analysis, under the hypothesis that common variants have an important role in the etiology of common diseases. These multilateral approaches identified *ATP2B1* as a gene responsible for hypertension in not only Japanese but also Caucasians. The high blood pressure susceptibility conferred by certain alleles of *ATP2B1* has been widely replicated in various populations. *Ex vivo* mRNA expression analysis in umbilical artery smooth muscle cells indicated that reduced expression of this gene associated with the risk allele may be an underlying mechanism relating the *ATP2B1* variant to hypertension. However, the effect size of a SNP was too small to clarify the entire picture of the genetic basis of hypertension. Further, dense genome analysis with accurate phenotype data may be required.
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Keywords: ATP2B1; high blood pressure; Millennium Genome Project

INTRODUCTION

In 2000, a number of national cooperative projects were started in Japan under the banner of 'Millennium Projects'. These projects aimed to achieve bold technological innovation in three areas of vital importance to Japan: information utilization, aging society and the environment. Among them, genome research in five fields of genetics, namely disease genes, human genome variation, the rice genome, bioinformatics, and development/differentiation/regeneration, was collectively termed the 'Millennium Genome Project (MGP)'. This project was launched to shed light on some of the unsolved challenges of the aging society by gaining an understanding of disease pathogenesis and by establishing genome-based personalized medical care (Figure 1).[1] In the Disease Genes division, the five most frequent diseases in developed countries that have a harmful impact on lifelong health and quality-of-life were selected, namely essential hypertension, diabetes, cancer, asthma and Alzheimer's disease. Among the study groups for these five target diseases, the group for hypertension consists of 16 researchers whose focus is the genetic aspects of essential hypertension (Figure 2). These researchers were selected from among scientists with established achievements in genetics and/or epidemiology of hypertension. The aim of this review is to summarize the results of gene hunting conducted by the study group for hypertension.

STUDY OVERVIEW

Two antithetical but complementary approaches were adopted to identify common variants associated with individual blood pressure susceptibility, a genetic statistics-based genome-wide approach and a knowledge-based candidate gene approach (Figure 3). A multi-stage genome-wide association study (GWAS) using approximately 80 000 gene-associated single-nucleotide polymorphisms (SNPs) was performed as a five study group initiative as the flagship study of the Disease Genes division. The Japanese-oriented SNPs (JSNPs) analyzed in this GWAS[2,3] and the high-throughput low-cost genotyping system based on the Invader technology were both developed by the Human Genome Variation division.

The study group for hypertension undertook a second GWAS using approximately 20 000 microsatellite (MS) markers with an average spacing of 146 kb. Although many studies have used commercially available MS marker sets consisting of <1000 markers to narrow down candidate gene loci, a Japanese research group has successfully identified 30 000 MS marker sets by systemic whole-genome screening and developed a high-throughput genotyping system based on the DNA-pooling method.[4]

The candidate gene approach was concomitantly designed and performed by each of the study groups. The study group for hypertension performed a systemic multiple candidate gene analysis,

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with candidate gene selection made from among genes related to signal transduction pathways, including receptors, soluble carrier proteins, binding proteins, channels, enzymes and G-proteins that were possibly related to blood pressure regulation.

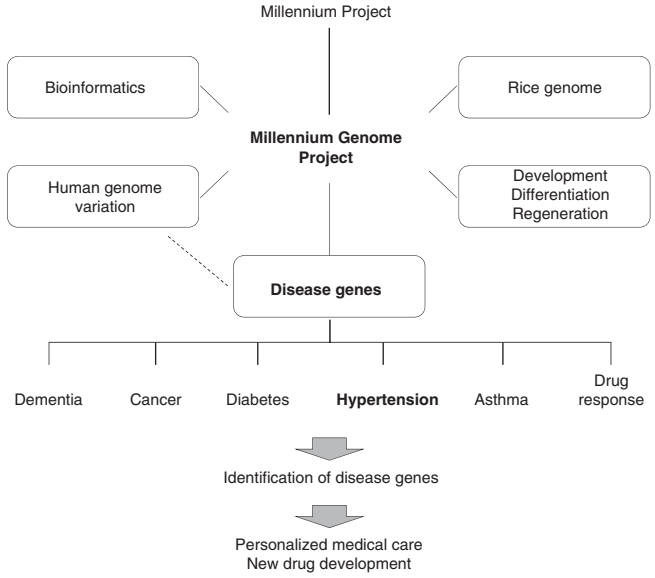

Figure 1 Overview of the MGP.

GWAS USING SNP MARKERS

To overcome financial and technological limitations, mostly owing to a lack of DNA chip technology, the Disease Genes division launched a coordinated two-stage-screening approach (Figure 4).[5] The theoretical basis of this exploratory test scheme has been reported elsewhere.[6] In the first stage, a total of 940 samples from 188 patients with one of the five target diseases were simultaneously genotyped, and the association analysis was performed using the case samples of the other four study groups as a pseudo control. Allele frequency data for 752 individuals from a general Japanese population established by the JSNP project were also used as a second control. Both this exploratory analysis as well as the second-stage analysis used cases of men with very severe hypertension gathered by the nationwide MGP collaboration. Among the 80 795 SNPs initially genotyped, the 2676 top-hit SNPs that showed an odds ratio (OR) >1.4 with a *P*-value <0.015 in at least one test comparison of allele frequency or genotype distribution were analyzed in the next stage analysis. A total of 75 SNPs that showed positive associations in the second-stage analysis, namely a *P*-value <0.01 for genotype distribution and <0.05 for allele frequency on comparison of 940 cases (188 first-stage cases and 752 second-stage cases) and 752 controls, were further analyzed in a second panel of 619 hypertensive subjects and 1406 normotensive controls. After the third-stage analysis, which was the study group's own extensive analysis, six SNPs located in four genes, *ADD2*, *EYA2*, *KIAA0789* and *M6PR*, were finally explored.

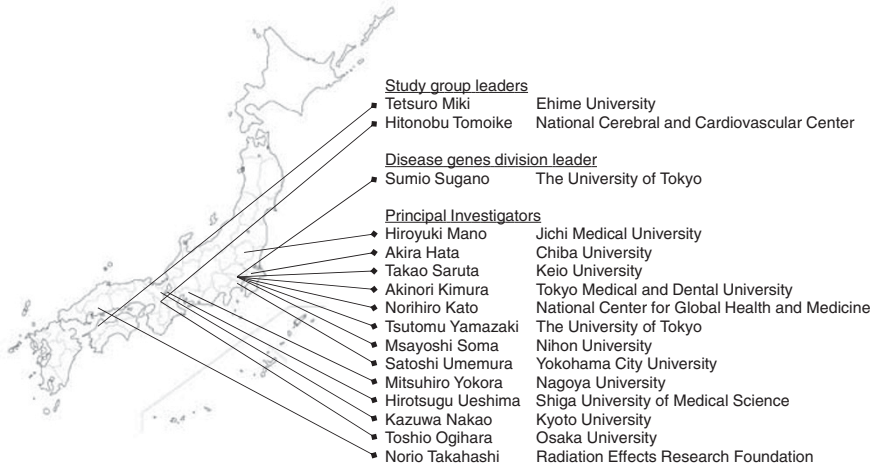

Figure 2 Investigators in the MGP for hypertension.

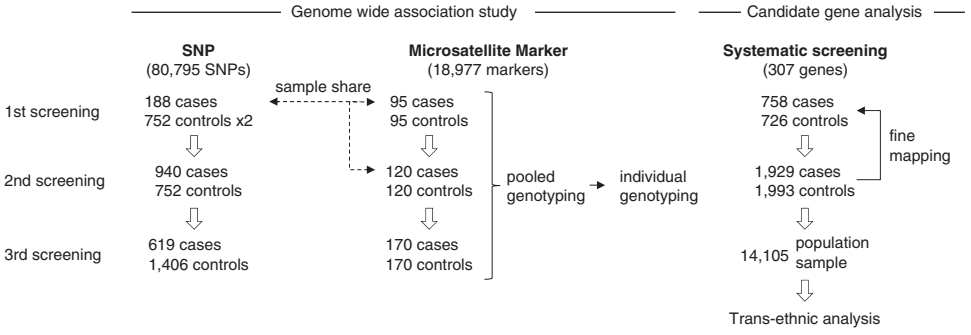

Figure 3 Overall study design of the MGP for hypertension.

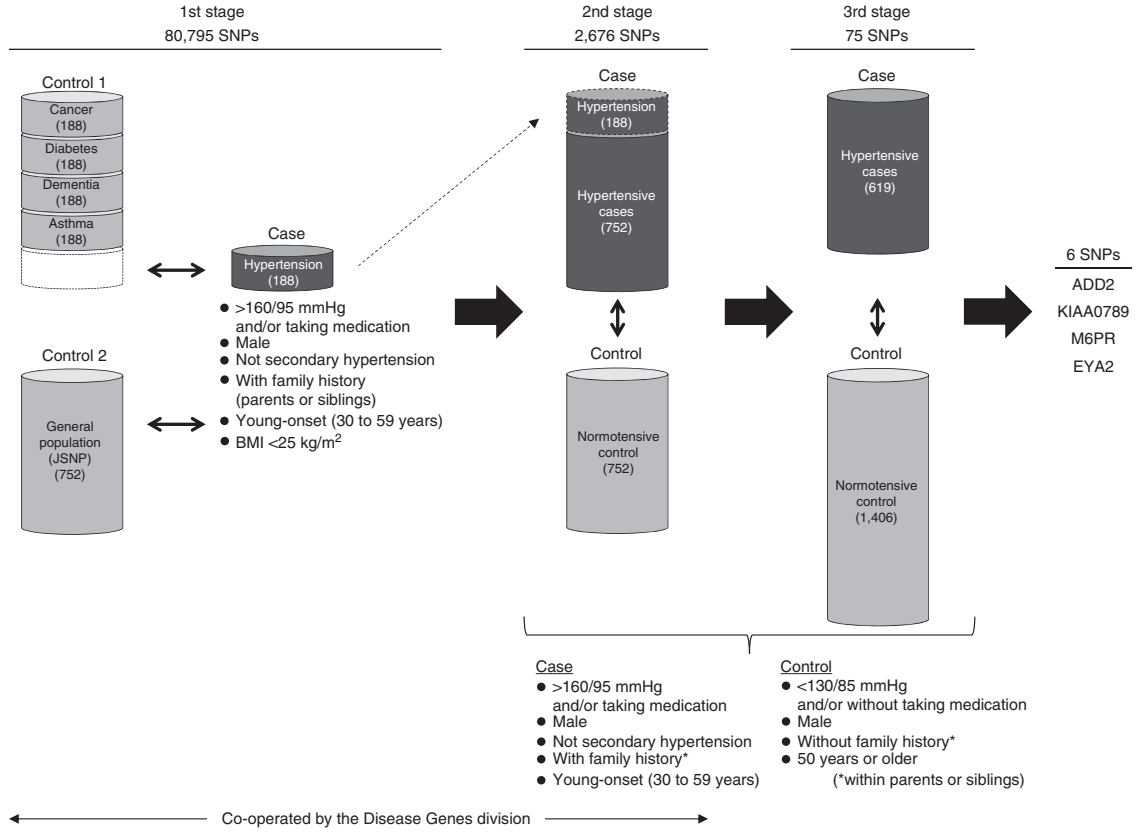

Figure 4 Schematic presentation of the three-stageSNP-based GWAS strategy.

Identification of the *ADD2* gene as a candidate locus for BP regulation is particularly noteworthy, because β-adducin, which is encoded by the *ADD2* gene, has been suggested to have a role in a physiological pathway of BP regulation.[7] Adducin is a heterodimeric cytoskeleton protein composed of α-, β- and γ-subunits, and is highly conserved across species. Point mutations on the *Add1* and *Add2* genes account for the 50% BP difference between Milan hypertensive and normotensive strains via the modulation of Na^+–K^+ activity.[8,9] In humans, association of the *ADD1* G460W polymorphism (rs4961) with hypertension has been widely investigated in various populations[7] under the hypothesis that the *ADD1* W allele accelerates Na^+–K^+ co-transport,[10] which in turn results in increased renal sodium reabsorption. As for the *ADD2* gene, several articles have reported that this may confer susceptibility in association analyses.[11,12] A linkage analysis also reported that the 2p13 region in *ADD2* was also a hypertension-related locus.[13] However, a recent large-scale GWAS in the European population,[14] as well as our study in East Asians,[15] found no SNPs of genome-wide significance near the *ADD2* gene, or the other three identified genes, namely *EYA2*, *KIAA0789* (renamed to *WSCD2*) and *M6PR*. Statistical power of previous GWAS was not enough to detect SNPs with smaller effect sizes. As larger GWAS samples can detect larger numbers of common susceptibility variants with smaller effects, increasing sample size may provide more convincing evidence.

GWAS USING MS MARKERS

A total of 385 hypertensive patients and 385 normotensive control subjects were analyzed in the three-stage screening using 18 977 MS markers (Figure 3).[16] A portionof the hypertensive cases analyzed in the first and second screenings were shared with the SNP-based GWAS. To bring down the cost and technical burden of genotyping 1000 MS markers, individual DNA samples of each stage were pooled within cases and controls. The pooled DNA was then amplified using the 18 977 MS markers, and the PCR products were genotyped by a standard protocol using capillary DNA sequencers. This DNA-pooling method enabled us to obtain the allele frequencies of MS markers in pooled Japanese individuals by measuring the height of multiple peaks. An association analysis was performed by comparing the height of peaks between the case and controls. *P*-values <0.05 were considered significant in all stage analyses to avoid potential false negatives.

After the three-stage analysis, 54 markers, which showed *P*-values <0.05 in either of the 2×2 (allelic) or $2 \times m$ (genotype, $m =$ number of alleles) analysis, were initially identified as potential susceptible loci for hypertension. These markers were again genotyped in all 770 individual samples (not pooled) used in the first- to third-stage analyses (385 cases and 385 controls), and finally 19 loci were identified. Among these 19 loci, three chromosomal locations, 6q27, 2p25.1 and 2p11.1-q12.3, overlapped with a region identified in other linkage[17,18] or admixture mapping studies.[19] Although these previous studies did not permit sufficient narrowing down of the region to allow a candidate gene to be identified, our dense mapping did allow the former two markers to be dropped into the *SMOC2* and *LPIN1* gene regions, respectively. Although the *SMOC2* gene encodes a matricellular protein that promotes matrix assembly and can stimulate endothelial cell proliferation and migration, an association between this gene and hypertension has not been suggested. Further, *LPIN1* is recognized as a candidate gene for human lipodystrophy,

which is characterized by a loss of body fat, fatty liver, hypertriglyceridemia and insulin resistance. Although one study reported replication data which suggested that one of the 12 tagged SNPs in the *LPIN1* gene region was a susceptibility locus for hypertension in Chinese men,[20] another study failed to replicate the associations.[21]

SYSTEMATIC CANDIDATE GENE SCREENING

Candidate gene analysis is the classic approach to find susceptible genotypes. Previous studies selected several candidate genes on the basis of known biochemical or physiologic components related to BP regulation. The results of previous studies which investigated a single or only a few arbitrarily selected genes have not been consistent, and positive findings have rarely been replicated. As hypertension is a polygenic disease, alleles at many uninvestigated loci would have contributed to the ultimate disease trait. We therefore conducted a systematic candidate gene screening by evaluating 307 genes selected from those related to signal transduction, binding proteins, channels, enzymes and G-proteins that were possibly related to BP regulation.[22,23]

The first screening was conducted using a case–control panel with 758 severe young-onset hypertensive patients and 726 strictly normotensive elderly persons enrolled from four independent regions throughout Japan (Table 1). One SNP per one gene, preferably located in the promoter region or exons with the highest minor allele frequency in the Japanese population, was selected based on the information published in the JSNP database (http://snp.ims.u-tokyo.ac.jp). Of the 307 SNPs analyzed, 38 showed *P*-values <0.05 in either analysis comparing allele frequency or genotype distribution, including a dominant or recessive model. To further identify susceptible SNPs, replication genotyping was performed using an independent nested population of 1929 cases and 1993 controls chosen from the approximately 14 000 cohort samples (Table 1). This cohort panel consist of six independent population- or company employee-based genetic epidemiological cohorts established by several of the members of the study group for hypertension. Results repeatedly showed the association of *ATP2B1* rs2070759 with hypertension (OR = 1.18, $P = 4.0 \times 10^{-4}$). The subsequent dense SNP analysis around the 150 kb region of the *ATP2B1* gene identified another significant SNP at the promoter region (rs11105378: OR = 1.31, $P = 4.1 \times 10^{-11}$), and the BP susceptibility of this SNP was replicated in the whole cohort sample analysis. Further, a trans-ethnic analysis using the Global BPgen data set,[24] a large-scale GWAS samples of European descent, confirmed that the *ATP2B1* gene was a susceptibility locus for hypertension (OR = 1.13, $P = 5.9 \times 10^{-4}$).

The per-allele effect size of rs11105378 for systolic BP trait was 1.33 mm Hg ($P = 1.5 \times 10^{-8}$) in the Japanese cohort sample. Meta-analysis with samples of the Global BPgen (0.59 mm Hg, $P = 0.001$) and the CHARGE[25] (1.31 mm Hg, $P = 9.1 \times 10^{-11}$) consortia allowed more precise estimation, at 1.02 mm Hg for systolic BP ($P = 1.4 \times 10^{-18}$) and 0.54 mm Hg for diastolic BP ($P = 1.6 \times 10^{-13}$). Further, combined analysis with other susceptible SNPs identified by recent GWAS in samples of European origin, namely *FGF5* rs1458038, *CYP17A1* rs1004467 and *CSK* rs1378942, showed a stepwise association with BP traits and hypertension risk. Individuals carrying seven or eight risk genotypes had higher systolic/diastolic BP (136.2/81.5 mm Hg), in contrast to the lower BP of individuals with only two or less-risk genotypes (125.3/57.5 mm Hg) (Figure 5). The OR of the high-risk group (OR = 1.43, $P = 1.0 \times 10^{-4}$) was 2.27 compared with the lowest risk group(OR = 0.63, $P = 0.020$).

In the SNP-based MGP-GWAS, two SNPs located in *ATP2B1* gene region, namely rs2070758 (TT/TG/GG = 153/34/1) and rs2070759 (AA/AC/CC = 49/102/35), were genotyped. However, in the association analysis with752 pseudo control samples, the *P*-value did not reach statistical significance (allele frequency; $P = 0.460$ and $P = 0.341$, respectively). MS markers located 2.4 kb upstream of the *ATP2B1* gene also did not show a positive association with hypertension. The most plausible reason for the failure to identify *ATP2B1* in the MGP-GWAS may be insufficient statistical power owing to a limited number of subjects. The susceptibility conferred by the *ATP2B1* gene is therefore clear, given the negative results of the MGP-GWAS.

Table 1 Study samples used in candidate gene screening

	Cohort name	*Origin*	N
Cohort panel ($n = 14\,105$)	Ohasama	General population	1592
	Yokohama	Company employees	2290
	Shigaraki	General population	2273
	Takashima	General population	1730
	Suita	General population	2536
	Matsuyama	Company employees	808
	Nomura	General population	2876
			Case/control
Case–control panel ($n = 1484$)	Asahikawa		192/192
	Tokyo		159/153
	Osaka		238/189
	Hiroshima		169/192

Hypertension cases: non-obese hypertensive patients who had a previous diagnosis of hypertension at between 30 and 59 years of age were either being treated with antihypertensive medication or had a SBP >160 mm Hg and/or DBP >100 mm Hg; and had a family history of hypertension in their parents and/or siblings. Normotensive controls: middle-aged to elderly subjects (aged >45 years), who had never been treated with antihypertensive medications; had a SBP <120 mm Hg and DBP <80 mm Hg; and had no family history of hypertension.

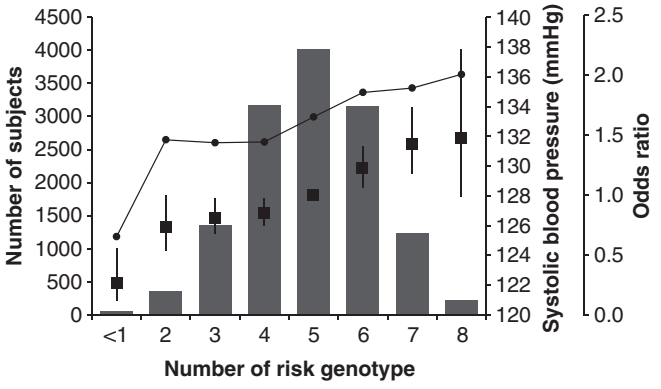

Figure 5 Adjusted OR for hypertension and systolic blood pressure by the number of risk genotypes. Number of risk genotypes was calculated by the following four SNPs: *ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1*, rs1004467 and *CSK* rs1378942. Age, age,[2] sex, BMI and cohort variable-adjusted OR and systolic BP (line graph) is shown in the panel. Number of subjects in each group is represented as a bar graph.

REPLICATION RESULTS OF THE BP SUSCEPTIBILITY OF ATP2B1

GWAS in people of European descent[25] and Koreans[26] also reported that the *ATP2B1* gene variants are the most significant determinants of BP traits and hypertension. The same results were also observed in another Japanese population.[27] A meta-analysis of the East-Asian GWAS data[28] further supported that the association was strongest for *ATP2B1*, except for the *ALDH2* rs671 genotype, which determines alcohol intolerance in East-Asians. A very recently published world-wide study for BP genetics[29] based on 270 000 persons in various populations, including our MGP data sets, provided conclusive evidence on this issue.

MECHANISMS OF THE ASSOCIATION BETWEEN ATP2B1 AND BP

The *ATP2B1* (so-called *PMCA1*) gene encodes the plasma membrane calcium ATPase isoform 1, which removes bivalent calcium ions from eukaryotic cells against very large concentration gradients and has a critical role in intracellular calcium homeostasis. Although the pathophysiological implications of *ATP2B1* gene products in the development of hypertension are uncertain, we obtained an important clue for the mechanism from an *ex vivo* expression analysis of *ATP2B1* mRNA. *ATP2B1* mRNA expression in umbilical artery smooth muscle cells differed among rs11105738 genotypes, and cells carrying the risk allele showed significantly lower levels of mRNA.[23]

In mammals, calcium ATPase isoforms are encoded by at least four separate genes (*ATP2B1–ATP2B4*).[30] Although we examined the possible association of *ATP2B4* gene polymorphisms with hypertension, the most promising of the four isoforms, we found no significant SNPs around the region, suggesting that the association of the plasma membrane Ca^{2+} pump with BP regulation is isoform-specific.

We recently reported[31] that the trimetric intracellular cation A (TRIC-A) channel knockout mouse showed markedly higher BP, and that several SNPs locating around the *TRIC-A* gene were significantly associated with hypertension in humans. TRIC channels are intracellular monovalent cation channels, which are postulated to mediate counter-ion movements facilitating physiological Ca^{2+} release from internal stores. It is particularly interesting that the two genes, which were identified by the independent two antithetical approaches of non-hypothesis statistic-based analysis (*ATP2B1*) and hypothesis-based analysis (*TRIC-A*), are molecules that play important roles in Ca^{2+} homeostasis (Figure 6). Dense SNP analysis or re-sequencing of other molecules relating to Ca^{2+} transport may further elucidate genetic factors for BP regulation.

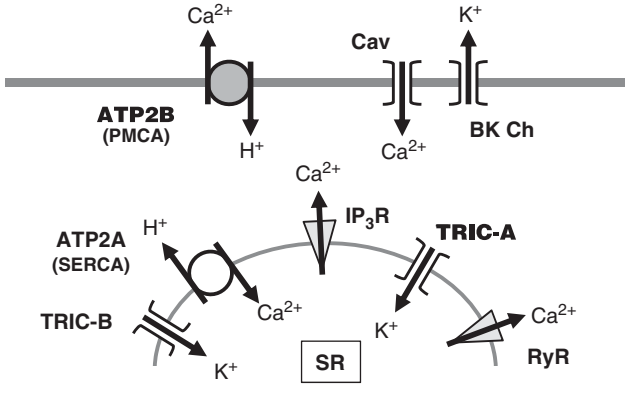

Figure 6 Molecules associated with Ca^{2+} movement at vascular smooth muscle cells. Cav, L-type Ca^{2+} channel; BK Ch, BK channel; IP_3R, inositol trisphosphophosphate receptor; RyR, ryanodine receptor.

PERSPECTIVES

Hypertension is one of the most prevalent complex genetic disorders, for which the genetic heritability has been estimated at up to 60%. Owing to its large impact on a number of cardiovascular diseases, hypertension is a major contributor to the global health burden. Identification of genetic variants associated with BP regulation is important in two aspects, recognition of genetically high-risk populations for effective prevention and early diagnosis and treatment, and elucidation of the molecular pathogenesis to identify target molecules for diagnosis, treatment and prevention. A series of genetic studies in the MGP for hypertension and recent GWAS in various populations identified several genes responsible for BP regulation. However, the effect size of each implicated SNP was small (<1 mm Hg), and the combination of all identified loci accounted for $<10\%$ of overall BP variation. As GWAS are firstly designed to identify common genetic variants for multifactorial diseases and have limited ability to detect less common or rare variants, missing heritability may be traced by analyzing the uncommon variants using a million chip based on the 1000G project data (www.1000genomes.org) or a next generation sequencer. BP is a phenotype that widely fluctuates as a result of non-genetic factors. Accurate BP measurement by ambulatory monitoring or home-measurement may also be indispensable for unlocking the genetic basis of hypertension.

CONFLICT OF INTEREST

The following authors 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: Yasuharu Tabara, Katsuhiko Kohara, Yoshikuni Kita, Nobuhito Hirawa, Jun Nakura, Satoshi Umemura, Hirotsugu Ueshima and Tetsuro Miki.

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APPENDIX

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