TCS C1784T (intron 1) in all subjects (CC:TT+CT=20:10 in NR, 40:5 in R, p=0.018, OR=4.00, CI:1.204-13.284, C allele:T allele=49:11 in NR, 85:5 in R, p=0.013, OR=3.81, CI:1.253-11.627). Thus, the newly detected *TCS C1784T* is a gene polymorphism susceptible to the antihypertensive effect of TZD in patients with essential hypertension.

In conclusion, SNPs analysis will provide a plausible tool for realizing tailor-made medicine in hypertension.

Introduction

The Millennium Genome Project was started in the year 2000 under a large delegation of three ministries including Health-Labor-Welfare, Education-Culture-Sports-Science-Technology and Economy-Trade-Industry. The final goal of this project is not only to realize "tailor-made medicine referencing each patient's genetic information," but also to find "seeds for new drugs" in common diseases including cancer, hypertension, diabetes mellitus, Alzheimer's disease and asthma. The National Cardiovascular Center (NCVC) was bound to control genetic studies on hypertension as one of the sub-leaders in the Millennium Genome Project and the studies were supported totally by a scientific grant of The Ministry of Health, Labor and Welfare. Several departments at NCVC collaborated in a consortium. Departments that participated were Preventive Cardiology, Hypertension-Nephrology, Atherosclerosis-Metabolism, Cardiology, Administration for bioinformatics, Etiology-Pathogenesis, Epidemiology and Bioscience. In this paper, we overview our approach and recent findings in the genetic analysis of human hypertension focusing on single nucleotide polymorphisms (SNPs) related to the effect of antihypertensive drugs at the Division of Hypertension and Nephrology in NCVC.

Genotyping methods

As an initial approach, the association study was performed using the candidate gene

approach. Regarding the selection of candidate genes, we conducted several processes to examine the association between hypertension and SNPs. One of the steps was hunting for SNPs following the direct sequencing of the promoter (regulatory SNPs: rSNP) and all coding regions of the genes (coding SNPs: cSNP). Genes known to have biochemical or physiological roles in the progression of hypertension were chosen, including genes related to the renin-angiotensin-aldosterone system (RAAS), vasoactive peptides, water-electrolyte metabolism and so on. As a consequence of hunting for SNPs for a gene, several SNPs would usually be detected because of their high prevalence in every several hundred bases, on average, throughout the human genome [1]. Thus, we choose SNPs for genotyping that have a minor allele frequency of at least above 5% or that induce nonsynonymous substitutions in the translated protein even if their minor allele frequency is less than 5% [2]. Previously, 525 genes were selected from the OMIM database of NCBI as candidate genes for hypertension by 19 key words related to blood pressure regulation. In total, 367 genes had been registered in the public database, SNPper. Nonsynonymous SNPs were mainly retrieved. Consequently, 201 out of the 367 genes retrieved had 675 cSNPs. We tried to sequence for these 675 SNPs using 32 healthy Japanese subjects. Finally, 525 cSNPs in 179 genes in total were successfully sequenced. We were able to confirm that 143 cSNPs of the 525 cSNPs

(27.2%) were already registered in the public database. We also found 16 new cSNPs which had not been registered in the public database, although these new SNPs were mostly rare (<5% in prevalence). Finally, 159 cSNPs were detected in the Japanese by sequencing the 525 cSNPs in the 179 hypertension candidate genes in the public database. Now, we are working on genotyping for these candidate SNPs with an above 5% allele frequency. We learned from this study that the public database of SNPs is a very important and useful resource, but it does not cover all SNPs. Therefore, we need to conduct own SNP search by sequencing for important genes that are suspected to be causative or relative genes of blood pressure regulation.

Furthermore, most SNPs often have linkage disequilibrium (LD) with others. Thus, we are using a representive SNP in the haplotype block with tight LD for the genotyping. We regard r-square≥0.5 as tight LD [3, 4]. It is known that the angiotension-converting enzyme (ACE) gene *I/D* polymorphism, which is a well-known gene polymorphism relating to cardiovascular diseases [4, 5], has LD with over 20 other polymorphisms [4]. Our recent study revealed the most suitable SNP, having tight LD with *ACE I/D* in the Japanese, for the genotyping, using the TaqMan PCR method [6] or invader method [7], which is commonly used as a typing method for SNPs. Public database db SNP ID: rs4341 is the G/C polymorphism in intron16. This SNP showed almost complete LD

with the *I/D* polymorphism. We genotyped the *ACE I/D* polymorphism in 511 Japanese subjects using conventional gel electrophoresis. It took about 2 days for genotyping in this manner. In contrast, it took only 2 hours for SNP rs4341 using the TaqMan PCR method. Moreover, only 6 ng of DNA was necessary for TaqMan PCR. This method was obviously time- and DNA-saving compared to genotyping by gel electrophoresis for *ACE I/D* polymorphism. Therefore, we are using the TaqMan PCR method for the genotyping of most of the selected SNPs in our project.

Clinical phenotypes and selection of candidate genes

The present protocol of SNPs analysis in hypertension was approved by the Millennium Genome Project's committee and the ethical committee in April, 2001. With written informed consent, we have collected DNA samples of patients with hypertension in our division. In this case-control study, all controls were sampled from the general population in Suita. [8]

To clarify the contribution of gene polymorphisms to hypertension, there are several important processes, which we should investigate regarding the contribution of genetic factors. These include 1) the relationships between genetic factors and initial mechanisms in blood pressure elevation, 2) genetic influences on the interaction

between environmental factors and blood pressure such as salt intake and low physical activity, 3) progression of hypertensive complications, and 4) sensitivity to antihypertensive drugs (Figure 1). All of these are regarded as important targets in the genetic study of hypertension. Thus, we first targeted the detection of SNPs related to the progression of hypertensive complications and the effect of antihypertensive drugs using about 1000 hypertensive subjects at the Division of Hypertension and Nephrology. Regarding the candidate genes, they were picked mostly from categories including RAAS, the sympathetic nervous system, vasoactive peptides and cytokines, water-electrolyte metabolism and ion-channels, oxidative stress, signal transduction, insulin resistance and so on. Many association studies on these candidate genes are now in progress for the Millennium Genome Project.

SNPs sensitive to the effect of antihypertensive drugs

The detection of gene polymorphisms susceptible to antihypertensive drugs is very important in realizing tailor-made medicine in the field of hypertension related diseases. It is well-known that African-American hypertensives with mostly low renin activity are resistant to RAAS inhibitors such as angiotensin-converting enzyme inhibitor (ACEI) and β-adrenergic receptor blocker, while they are sensitive to thiazide diuretics [9].

To investigate the gene polymorphisms susceptible to antihypertensive drugs, two approaches have been mainly performed so far. One is the pharmacokinetic approach focusing on drug metabolism. It is well recognized that the CYP2D6 genetic polymorphisms are related to the effects of various drugs including β-adrenergic receptor blockers [10]. Another is the pharmacodynamic approach targeting genes relevant to the pharmacologic activities of drugs [11] . An antihypertensive drug lowers blood pressure by acting on specific targets, which modulate intermediate systems to affect the tone of vascular smooth muscle, the level of blood volume, the activities of autonomic nervous systems and so on. Since many components of the systems are proteins that may vary in structure, configuration, or quantity because of genetic differences among individuals, it is reasonable to expect that inter-individual variation in blood pressure responses to these drugs would be, in part, genetically determined. Obvious candidate genes for influencing blood pressure responses would code for components of a system targeted by the drug. Additional candidates are genes that code for components of the counter-regulatory systems opposing an initial drug-induced fall in blood pressure.

There have been several reports [12-19] investigating the gene polymorphisms relating to the effects of antihypertensive drugs (Table). We designed association studies to find

SNPs susceptible to the antihypertensive effect of three important antihypertensive drugs, thiazide diuretics (TZD) [20], dihydropyridine calcium channel blocker (CCB) [21] and ACEI [22].

Gene polymorphism susceptible to the effect of thiazide diuretics

TZD are very useful antihypertensive agents for salt-sensitive and low-renin patients with hypertension [23]. Low-dose TZD are recommended as the most considerable first-choice antihypertensive drug in the Joint National Committee VII guideline [23]. However, the long-term use of TZD sometimes induces abnormalities of glucose and lipid metabolism. Therefore, prediction of the antihypertensive effect of TZD could be applicable for tailor-made medicine in hypertension.

Turner et al. [13] reported that the β3-subunit of G protein (*GNB3*) *C825T* polymorphism was related to the antihypertensive effect of TZD in both Caucasian and African-American subjects with essential hypertension. Glorioso et al. [24] also demonstrated that the α-adducin (*ADD1*) *Gly460Trp* polymorphism would be the susceptible gene to the antihypertensive effect of TZD in Italian hypertensives. This *ADD1 Gly460Trp* polymorphism was also suggested to be a salt-sensitive susceptible gene polymorphism in essential hypertension in Caucasians and Asians [25].

Causative genes were recently detected in several monogenic electrolyte disorders,

such as Gitelman syndrome [26], Gordon syndrome (pseudohypoaldosteronism type II) [27] and pseudohypoaldosteronism type I (PHA I). Gitelman syndrome, an autosomal recessive disorder known as a variant form of Bartter's syndrome that has clinical features including sodium wasting, low blood pressure, secondary hyperaldosteronism, hypokalemia, alkalosis, hypomagnemia, and hypocalciuria, is due to mutations of the thiazide-sensitive Na-Cl cotransporter (*TSC*) gene [28]. Gordon syndrome, an autosomal dominant genetic disorder clinically characterized by hypertension, metabolic acidosis and high serum chloride, is caused by mutation of the *WNK1* and *4* genes [27]. We recently found novel missense mutations in WNK4, which may be related to the severity of hypertension [29]. TZD are commonly effective for these two syndromes. Furthermore, we focused on the Na^+/Ca^{2+} exchanger gene (*NCX1*), because its impairment was recently reported in mesangial cells of salt-sensitive hypertensive rats [30]. To detect the SNPs susceptible to the antihypertensive effect of TZD, we focused on genes related to water-electrolyte handling in the kidney.

Seventy-six outpatients (mean age 65.3±1.0 y.o.) with essential hypertension taking TZD either as monotherapy or combination therapy with other agents were assessed retrospectively [20]. We defined as responders (R) any patients whose mean blood pressure was lowered by over 5 mmHg after TZD treatment. Genotyping using the

TaqMan PCR method for *thiazide sensitive Na-Cl cotransporter* (*TSC* : 12 SNPs detected by direct sequences), *alpha-adducin* (*Gly460Trp*), Na^+/Ca^{2+} *exchanger* (*NCX1* : 7 SNPs), *WNK1* (7 SNPs), *WNK4* (2 SNPs) and, G protein subunit (*GNB3-s* : *C825T*) was performed. The SNPs of *TSC, NCX1, WNK1 and WNK4* were detected by the direct sequence. All allele frequencies were over 5%.

The comparison of polymorphisms prevalence between R and non-responders (NR) showed a significant difference only in *TCS C1784T* (intron 1) in all subjects (CC:TT+CT=20:10 in NR, 40:5 in R, p=0.018, OR=4.00, CI:1.204-13.284, C allele:T allele=49:11 in NR, 85:5 in R, p=0.013, OR=3.81, CI:1.253-11.627). The newly detected *TCS C1784T* is a gene polymorphism susceptible to the antihypertensive effect of TZD in patients with essential hypertension. Thus, the prediction of blood pressure reduction by TZD by evaluating *TCS C1784T* may be possible (Figure 2).

By the same methods, we clarified the C/T polymorphisms in intron 1 of *NCX1* (*T-23200C*) as the gene polymorphisms susceptible to dihydropyridine CCBs [21].

Furthermore, *Lys198Asn* in exon 5 of the endothelin-1 gene (*EDN1*) which was associated with hypertension in overweight subjects [31], was elucidated as the gene polymorphism susceptible to ACEI in females [22] (Figure 2).

These gene polymorphisms susceptible to the antihypertensive effects of TZD, CCB

and ACEI would be very useful in realizing tailor-made medicine for hypertension based on genetic background.

Conclusion

This paper summarized our approaches of genetic analysis in hypertension to realize tailor-made medicine. Since hypertension is a multi-factorial disease, genetic influences would not be so much on the etiology of hypertension [5]. However, we consider that genetic factors influence the various process of blood pressure elevation such as salt sensitivity and weight gain, and genetic factors are also related to the efficacy of various treatments of hypertension including pharmacological and non-pharmacological therapies. We would try to clarify the overall genetic influences in hypertension and confirm Tailor-made medicine for hypertension based on genetic background.

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References

1. Kruglyak L.: Prospects for whole-genome linkage disequilibrium mapping of common disease genes. *Nat Genet* 22:139-144, 1999

2. Okuda T, Fujioka Y, Kamide K, Kawano Y, Goto Y, Yoshimasa Y, Tomoike H, Iwai N, Hanai S, Miyata T.: Verification of 525 coding SNPs in 179 hypertension candidate genes in the Japanese population: identification of 159 SNPs in 93 genes. *J Hum Genet* 47:387-394, 2002

3. Ardlie KG, Kruglyak L, Seielstad M.: Patterns of linkage disequilibrium in the human genome. *Nat Rev.* 3:299-309., 2002

4. Rieder MJ, Taylor SL, Clark AG, Nickerson DA.: Sequence variation in the human angiotensin converting enzyme. *Nat Genet* 22:59-62, 1999

5. Kato N.: Genetic analysis in human hypertension. *Hypertens Res* 25:319-327, 2002

6. Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, Miyata T.: An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 26:301-306, 2003

7. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, Sato H, Sato H,

Hori M, Nakamura Y, Tanaka T.: Functional SNPs in the lymphotoxin- gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 32:650-654, 2002

8. Mannami T, Konishi M, Baba S, Nishi N, Terao A.: Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city. The Suita Study. *Stroke* 28:518-525, 1997

9. Jamerson K, DeQuattro V.: The impact of ethnicity on response to antihypertensive therapy. *Am J Med* 101:22S-32S, 1996

10. Linder MW, Prough RA, Valdes R Jr.: Pharmacogenetics-a laboratory tool for optimizing therapeutic efficacy. *Clin Chem* 43:254-266, 1997

11. Turner ST, Schwartz GL, Chapman AB, Hall WD, Boerwinkle E.: Antihypertensive pharmacogenetics: getting the right drug into the right patient. *J of Hypertension* 19:1-11, 2001

12. Glorioso N, Manunta P, Filigheddu F, Troffa C, Stella P, Barlassina C, Lombardi C, Soro A, Dettori F, Parpaglia PP, Alibrandi MTS, Cusi D, Bianch G.: The role of α-adducin polymorphism in blood pressure and sodium handling regulation may not be exclude by a negative association study. *Hypertension* 34:649-654,

1999

13. Turner ST, Schwartz GL, Chapman AB, Boerwinkle E.: C825T polymorphism of the G protein β3-subunit and antihypertensive response to a thiazide diuretic. *Hypertension* 37:739-743, 2001

14. Jia H, Hingorani AD, Sharma P, Hopper R, Dickerson C, Trutwein D, Lloyd DD, Brown MJ.: Association of the G(s)α gene with essential hypertension and response to β-blockade. *Hypertension* 34:8-14, 1999

15. O'Shaughnessy KM, Fu B, Dickerson C, Thurston D, Brown MJ.: The gain-of-function G389R variant of the β1-adrenoceptor does not influence blood pressure or heart rate response to β-blockade in hypertensive subjects. *Clinical Science* 99:233-238, 2000

16. Dudley C, Keavney B, Casadei B, Conway J, Bird R, Ratcliffe P: Prediction of patient responses to antihypertensive drugs using genetic polymorphisms: investigation of renin-angiotensin system genes. *J Hypertens* 14:259-262, 1996

17. Hingorani AD, Jia H, Stevens PA, Hopper R, Dickerson JEC, Brown M.: Renin-angiotensin system gene polymorphism influence blood pressure and the response to angiotensin converting enzyme inhibition. *J Hypertens* 13:1602-1609, 1995

18. Sasaki M, Oki T, Iuchi A, Tabata T, Yamada H, Manabe K, Fukuda K, Abe M, Ito S.: Relationship between the angiotensin converting enzyme gene polymorphism and the effects of enalapril on left ventricular hypertrophy and impaired diastolic filling in essential hypertension: M-mode and pulsed Doppler echocardiographic studies. *J Hypertens* 14:1403-1408, 1996

19. Kurland L, Melhus H, Karlsson J, Kahan T, Malmqvist K, Ohman P, Nystrom F, Hagg A, Lind L: Angiotensin converting enzyme gene polymorphism predicts blood pressure response to angiotensin II receptor type 1 antagonist treatment in hypertensive patients. *J Hypertens* 19:1783-1787, 2001

20. Matayoshi T, Kamide K, Takiuchi S, Yoshii M, Miwa Y, Takami Y, Tanaka C, Banno M, Horio T, Miyata T, Kawano Y.: Susceptible gene polymorphisms to the antihypertensive effect of thiazide diuretics. *Am J Hypertens* 16:18A, 2003

21. Yoshii M, Takiuchi S, Kamide K, Miwa Y, Matayoshi T, Takami Y, Horio T, Tanaka C, Banno M, Iwamoto T, Shigekawa M, Miyata T, Kawano Y.: Polymorphism of Na^+/Ca^{2+} exchanger gene and antihypertensive response to calcium channel blockers in patients with essential hypertension. *Am J Hypertens* 16:18A, 2003

22. Kamide K, Takiuchi S, Takami Y, Yoshii M, Matayoshi T, Miwa Y, Tanaka C,

Banno M, Horio T, Miyata T, Kawano Y.: Susceptible gene polymorphism to the antihypertensive effect of angiotensin converting enzyme inhibitors. *Am J Hypertens* 16:80A, 2003

23. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, Jones DW, Materson BJ, Oparil S, Wright JT Jr, Roccella EJ; National Heart, Lung, and Blood Institute Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure; National High Blood Pressure Education Program Coordinating Committee.: The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. *JAMA* 289:2560-2572, 2003

24. Glorioso N, Filigheddu F, Troffa C, Stella P, Barlassina C, Lombardi C, Soro A, Dettori F, Parpaglia PP, Alibrand MTS, Cusi D, Bianchi G.: The role of _-adducin polymorphism in blood pressure and sodium handling regulation may not be excluded by a negative association study. H*ypertension* 34:649-654, 1999

25. Sugimoto K, Hozawa A, Katsuya T, Matsubara M, Ohkubo T, Tsuji I, Motone M, Higaki J, Hisamachi S, Imai Y, Ogihara T.: Alpha-adducin Gly460Trp polymorphism is associated with low renin hypertension in younger subjects in

the ohasama study. *J Hypertens* 20:1779-1784, 2002

26. Gitelman HJ, Graham JB, Welt LG..: A new familial disorder characterized by hypokalemia and hypomagenesemia. *Trans Assoc Am Physicians* 79:221-235, 1966

27. Wilson FH, Disse-Nicodeme S, Choate KA, Ishikawa K, Nelson-Williams C, Desitter I, Gunel M, Milford DV, Lipkin GW, Achard J-M, Feely MP, Dussol B, Berland Y, Unwin RJ, Mayan H, Simon DB, Farfel Z, Jeunemaitre X, Lifton RP.: Human hypertension caused by mutations in WNK kinases. *Science* 293:1107-1112, 2001

28. Simon DB, Nelson WC, Bia MJ, Ellison D, Karet FE, Molina AM, Vaara I, Iwata F, Cushner HM, Koolen M, Gainza FJ, Gitleman HJ, Lifton RP.: Gitleman's variant of Bartter's syndrome, inherited hypokalemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* 12:24-30, 1996

29. Kamide K, Takiuchi S, Tanaka C, Miwa Y, Yoshii M, Horio T, Mannami T, Kokubo Y, Tomoike H, Kawano Y, Miyata T.: Three novel missense mutations of WNK4, a kinase mutated in inherited hypertension, in Japanese hypertensives: Implication of clinical phenotypes. *Am J Hypertens,* 2004 (in

press)

30. Hwang EF, Williams I, Kovacs G, Peti-Peterdi J, Siroky B, Rice WC, Bates E, Schwiebert EM, Unlap MT, Bell PD.: Impaired ability of the Na^+/Ca^{2+} exchanger from the Dahl/Rapp salt sensitive rat to regulate cytosolic calcium. *Am J Physiol Renal Physiol,* 2003

31. Tiret L , Poirier O, Hallet V, McDonagh TA, Morrison C, McMurray JJ, Dargie HJ, Arveiler D, Ruidavets JB, Luc G, Evans A, Cambien F.: The Lys198Asn polymorphism in the endothelin-1 gene is associated with blood pressure in overweight people. *Hypertension* 33:1169-1174, 1999

Table. Reported candidate gene studies of blood pressure response to antihypertensive drug therapy

Antihypertensive drug	Gene	Polymorphism	Allele association	Subjects	Reference
Thiazide diuretics	Adducin	*Gly460Trp*	Positive	Essential HT N=143 Caucasians	12
	G protein β_3 subunit	*C825T*	Positive	Essential HT N=190 Caucasians	13
β blocker	Gs protein α subunit	*FokI* (+/–)	Positive	Essential HT N=197 Black	14
	β_1 adrenoreceptor	*Gly389Arg*	Negative	Essential HT N=66 Caucasians	15
	Angiotensinogen	*Met235Thr*	Negative	Essential HT N=92,55 Caucasians	16
ACEI	ACE	*Insertion/Deletion*	Negative	Essential HT N=63-91 Caucasians	16
			Negative	Essential HT N=63-91 Caucasians	17
			Negative	Essential HT N=125 Caucasians	18
	Angiotensinogen	*Met235Thr*	Positive	Essential HT N=60 Japanese	17
			Negative	Essential HT N=125 Caucasians	16
	Angiotensin II type 1 receptor	*A1166C*	Negative	Essential HT N=63-91 Caucasians	17
ARB	ACE	*Insertion/Deletion*	Positive	Essential HT N=125 Caucasians	19
dCCB	ACE	*Insertion/Deletion*	Negative	Essential HT N=86 Caucasians	16
	Angiotensinogen	*Met235Thr*	Negative	Essential HT N=63-91 Caucasians	16

HT: hypertension, ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin II type 1 receptor blocker, dCCB: dihydropyridine calcium channel blocker