Table 4 Clinical profiles of 11 subjects with missense/frameshift mutations in the general population

Individual	1	2	3	4	5	6	7	8	9	10	11
Mutation	Q2R	Q2R	Q2R	R44H	R44H	R44H	R44H	R44H	Q78H	Q78H	1925–1926insT
Age (years)	61	83	73	58	71	81	80	61	52	53	59
Sex	Female	Female	Male	Female	Male	Male	Male	Male	Female	Male	Female
Body mass index (kg/m^2)	18.0	28.1	25.6	24.0	25.4	24.0	20.5	17.7	21.0	25.8	28.0
Systolic blood pressure (mmHg)	127	171	166	143	152	131	145	131	127	110	120
Diastolic blood pressure (mmHg)	72	67	83	86	82	78	92	83	80	75	63
Total cholesterol (mg/dl)	236	237	232	183	242	187	235	217	213	171	251
HDL-cholesterol (mg/dl)	61	65	65	56	44	69	73	53	59	46	57
Triglyceride (mg/dl)	125	76	57	104	292	55	72	112	137	95	104
Creatinine (mg/dl)	0.4	0.6	0.7	0.4	0.9	0.9	0.7	0.8	0.7	0.9	0.6
Overt proteinuria	–	–	–	–	–	2+	–	–	–	–	–
Fasting blood sugar (mg/dl)	81	109	87	147	92	104	96	83	86	101	145
Haemoglobin A1c (%)	5.3	5.6	5.7	6.9	5.4	5.9	5.4	5.4	5.2	5.6	6.4
Current smoker	No	No	Yes	No	Yes	Yes	Yes	No	Yes	No	No
Current drinker	No	No	No	No	No	No	No	No	No	No	No
Hypertension	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes
Hyperlipidemia	Yes	Yes	Yes	No	Yes	No	Yes	Yes	No	No	Yes
Diabetes mellitus	No	Yes	Yes	Yes	No	Yes	No	No	No	Yes	Yes
Other diseases		Stroke				Stroke					
Antihypertension treatment	No	No	Yes	No	Yes	Yes	No	No	No	No	Yes

Hypertension indicates systolic blood pressure of 140 mmHg or greater and diastolic blood pressure of 90 mmHg or greater, or antihypertensive medication. Hyperlipidemia, total cholesterol of 220 mg/dl or greater, or antihyperlipidemia medication. Diabetes, fasting plasma glucose of 126 mg/dl or greater, or non-fasting plasma glucose of 200 mg/dl or greater, or haemoglobin A1c of 6.5% or greater, or antidiabetic medication. HDL, high-density lipoprotein.

lipoprotein (HDL)-cholesterol, and the percentage of hyperlipidemia were significantly higher in women than in men. In this population, 771 individuals were diagnosed with hypertension.

In the general population, four (Q2R $n = 3$, R44H $n = 5$, Q78H $n = 2$, 1925–1926insT $n = 1$) out of six missense/frameshift mutations were observed among 11 subjects (Table 4). The remaining two missense mutations (Q2L, M5V) were not present in the general population, suggesting that Q2L and M5V might be specific for hypertensive patients (Table 5). The three mutations, Q2R, R44H, and Q78H, were found in both hypertensive and normotensive subjects (Table 5). Six out of seven individuals with the R44H mutation had hypertension (Table 5). The remaining 1925–1926insT mutation was observed in one individual (case 11) who was diagnosed with essential hypertension, NIDDM, and hyperlipidemia (Table 4). Therefore, four rare mutations, Q2L, M5V, R44H, and 1925–1926insT, are probably linked with hypertension, and the 1925–1926insT mutation was associated with various phenotypes, including hypertension, hyperlipidemia, and diabetes mellitus.

Table 5 Number of subjects with missense/frameshift mutations in the hypertensive and general populations

		General population	
Mutations	Hypertensive population ($n = 953$)	Hypertensive subjects ($n = 771$)	Normotensive subjects ($n = 1102$)
Q2L	2	0	0
Q2R	1	2	1
M5V	1	0	0
R44H	2	4	1
Q78H	1	0	2
1925–1926insT	1	1	0
Total	8	7	4

Association of common single-nucleotide polymorphisms with hypertension and diabetes mellitus

Next, we looked for an association in the general population between common SNP in *RGS2* and hypertension, diabetes mellitus or hyperlipidemia. Three common SNP, −638A > G, 1026T > A, and 1891–1892delTC, were genotyped for the association in population-based samples. These three SNP were in linkage disequilibrium with an r-square more than 0.5. The results of the case–control study are shown in Table 6. The 1891–1892delTC polymorphism was significantly associated with hypertension in women ($\chi^2 = 6.34$, $P = 0.04$). Multivariate logistic regression analysis performed after adjusting for age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) showed a significant association of two common SNP, 1026T > A [TT versus TA + AA: odds ratio (OR) 1.33; 95% confidence interval (CI) 1.02–1.74; $P = 0.035$] and 1891–1892delTC (I: insertion allele, D: deletion allele, II versus ID + DD: OR 1.47; 95% CI 1.09–1.97; $P = 0.012$), with hypertension in women (Table 7). A significant association of 1891–1892delTC (II versus ID + DD: OR 1.23; 95% CI 1.02–1.50; $P = 0.034$) with hypertension was observed in total. However, no SNP were associated with diabetes mellitus and hyperlipidemia (data not shown).

Discussion

Previous studies showed that mice deficient in *Rgs2* exhibited a strong hypertensive phenotype and persistent constriction of the resistance vasculature, implicating *RGS2* as a candidate gene for hypertension in humans [16,17]. In our study, we evaluated the relationship between genetic polymorphisms in *RGS2* and clinical phenotypes such as hypertension using two different populations, a hypertensive population and the general

Table 6 Genotype distributions of three polymorphisms of *RGS2* in normotensive and hypertensive individuals

		Women		Men		Total	
Polymorphisms	Genotypes	Hypertensives *n* (%)	Normotensives *n* (%)	Hypertensives *n* (%)	Normotensives *n* (%)	Hypertensives *n* (%)	Normotensives *n* (%)
−638A > G	AA	102 (27)	197 (31)	106 (27)	131 (28)	208 (27)	328 (30)
	AG	183 (49)	320 (50)	206 (52)	234 (50)	389 (50)	554 (50)
	GG	89 (24)	120 (19)	85 (21)	100 (22)	174 (23)	220 (20)
			$\chi^2 = 3.95$, $P = 0.14$		$\chi^2 = 0.27$, $= 0.87$		$\chi^2 = 2.70$, $P = 0.26$
1026T > A	TT	126 (34)	257 (40)	136 (34)	167 (36)	262 (34)	424 (39)
	TA	193 (52)	290 (46)	197 (50)	221 (48)	390 (51)	511 (46)
	AA	55 (14)	90 (14)	64 (16)	77 (16)	119 (15)	167 (15)
			$\chi^2 = 4.63$, $P = 0.10$		$\chi^2 = 0.39$, $P = 0.82$		$\chi^2 = 4.19$, $P = 0.12$
1891–1892delTC	II	122 (33)	257 (40)	135 (34)	163 (35)	257 (33)	420 (38)
	ID	196 (52)	288 (45)	195 (49)	225 (48)	391 (51)	513 (47)
	DD	56 (15)	92 (15)	67 (17)	77 (17)	123 (16)	169 (15)
			$\chi^2 = 6.34$, $^*P = 0.04$		$\chi^2 = 0.10$, $P = 0.95$		$\chi^2 = 4.61$, $P = 0.10$

n, Number of individuals; %, frequency of each genotype; $^*P < 0.05$.

population. The sequencing and genotyping of six rare missense/frameshift mutations in both populations indicated that three mutations, Q2L, M5V, and 1925–1926insT, might be specific for the hypertension phenotype. Six out of seven individuals with another of the mutations, R44H, had hypertension, also strongly linking this mutation to the hypertensive phenotype (Table 5). In addition, two common SNP in *RGS2* were associated with hypertension in women from the general population (Tables 6 and 7). Taken together, these data suggest that *RGS2* is involved in hypertension in humans.

Like other members of the RGS family, RGS2 regulates G protein signaling partly by acting as a guanosine triphosphatase-activating protein for several classes of Gα subunits via its core RGS domain [25,26]. The binding of RGS proteins to active Gα subunits can also interfere with effector binding, thereby blocking activation and downstream signaling [27]. The core domain of rat RGS4 has been defined as K58–T178 by X-ray crystallography [28]. From the amino acid sequence alignment of rat RGS4 with human RGS2, the human RGS2 core domain is predicted to extend from L79 to C199. Four of the missense mutations identified in this study (Q2L, Q2R, M5V, R44H) are not present in the RGS2 core domain, but the remaining mutation, Q78H, is very close to the core domain. As shown in Figure 1a, four of the missense mutations occurred in highly conserved residues among three different species, indicating that these mutations may result in functional changes in RGS2.

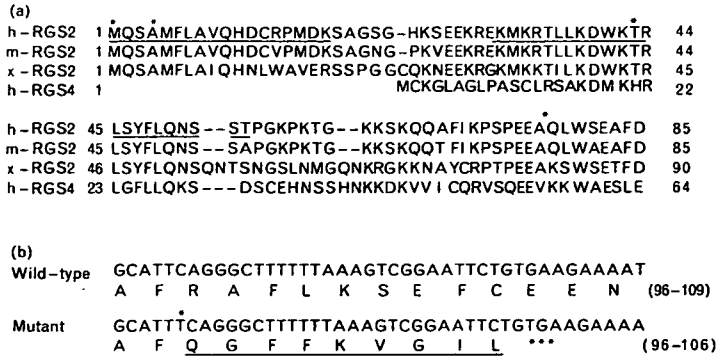
RGS2 shares with RGS4 and RGS16 a conserved *N*-terminal domain that is necessary and sufficient for plasma membrane targeting [29]. Importantly, it was recently reported that RGS2 directly binds to the C1 domain of type V adenylyl cyclase, inhibiting the activity of this enzyme, and that the *N*-terminal 19 amino acid residues of RGS2 are sufficient for this inhibitory effect [30]. Three mutations, Q2L, Q2R, and M5V, are present in this domain, suggesting functional changes in mutation-bearing RGS2 mutants. We identified two missense mutations, Q2L and M5V, in RGS2 in the hypertensive population but not in the general population, implying that these mutations might be involved in

Table 7 Comparison of hypertension prevalence by genotypes of three polymorphisms of *RGS2*

		Women		Men		Total	
Polymorphisms	Genotypes	Odds ratio (95% CI)	*P**	Odds ratio (95% CI)	*P**	Odds ratio (95% CI)	*P**
−638A > G	AA	1		1		1	
	AG + GG	1.21 (0.90–1.58)	0.219	0.94 (0.68–1.29)	0.692	1.20 (0.97–1.46)	0.091
	AA + AG	1		1		1	
	GG	1.23 (0.85–1.70)	0.266	0.89 (0.63–1.26)	0.51	1.04 (0.76–1.36)	0.912
1026T > A	TT	1		1		1	
	TA + AA	1.33 (1.02–1.74)	0.035	1.07 (0.81–1.42)	0.858	1.21 (0.98–1.47)	0.078
	TT + TA	1		1		1	
	AA	1.05 (0.73–1.51)	0.8	0.97 (0.67–1.39)	0.671	1.10 (0.88–1.50)	0.415
1891–1892delTC	II	1		1		1	
	ID + DD	1.47 (1.09–1.97)	0.012	0.96 (0.71–1.29)	0.747	1.23 (1.02–1.50)	0.034
	II + ID	1		1		1	
	DD	1.07 (0.72–1.61)	0.726	0.98 (0.72–1.47)	0.901	1.05 (0.81–1.35)	0.717

CI, Confidence interval; D, deletion allele; I, insertion allele. *Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) for hypertension.

Fig. 1



Partial amino acid sequence surrounding the mutations in RGS2. (a) Alignment of partial amino acid sequences of RGS2 from three species and human RGS4. RGS2 sequences are from *Homo sapiens* (h), *Mus musculus* (m), and *Xenopus laevis* (x). Numbers indicate the position of the amino acid sequence. The asterisks indicate the positions at which missense mutations occur (Q2L, Q2R, M5V, R44H, Q78H). Underlining in the human RGS2 sequence indicates the *N*-terminal 19 amino acid residues and the amphipathic α-helical domain. (b) Nucleotide and amino acid sequences of wild-type allele and the 1925–1926insT allele. Numbers indicate the amino acid residues. The asterisk indicates 1925–1926insT, which causes a frameshift mutation from Q98. The extended nine amino acids are underlined. ***Stop codon.

the pathogenesis of hypertension. However, as most of the hypertensive patients were treated, it is difficult to clarify the relationship of the severity of hypertension with the mutations. Further in-vitro and in-vivo studies are needed to clarify whether these missense mutations really result in functional changes in RGS2. The pedigrees of carriers harbouring these mutations would be useful for the co-segregation analysis with hypertension.

The importance of an amphipathic α-helical domain consisting of 23 amino acid residues from K32 to T54 in RGS2 has been proposed [29]. This helix forms a hydrophobic face (W41, L45, F48, L49) surrounded by two positive charges, K42 and R44, and this amphipathic structure is essential for the plasma membrane localization of RGS2 [29]. The R44H mutation is contained within this amphipathic α-helical domain and may thereby interfere with the membrane association of RGS2. We identified seven individuals with this mutation (Tables 2, 4 and 5), of whom six showed hypertension. The predicted functional defect as well as the high prevalence among hypertensive individuals suggests that the R44H mutation may be linked with hypertension in our population. The frequency of the mutant allele is calculated to be 0.133% (five alleles/3746 alleles) in the Japanese general population. The functional effect of this mutation needs to be examined experimentally.

We identified two hypertensive patients with a frameshift mutation (1925–1926insT) in RGS2. It is evident that this mutation results in the dysfunction of RGS2 (Fig. 1b). One of these two patients with 1925–1926insT had specific clinical phenotypes that were not only high blood pressure but also resistance to the ARB, losartan. The allele frequency of this mutant is very low (0.027%, one allele/3746 alleles) in the Japanese general population. However, it is worthwhile noting that this defective allele might be rich in other ethnic populations, because the frequency of some genetic mutations varies with ethnicity. Rare genetic mutations collectively contributing to a quantitative trait variation, such as plasma levels of HDL-cholesterol, have recently been reported [31]. In this scenario, the frameshift mutation and three of the missense mutations, Q2L, M5V, and R44H, in RGS2 could contribute collectively to hypertension (Table 5). Although only 0.86% of hypertensive subjects had missense/frameshift mutations in the two populations studied here, we have previously identified missense mutations in hypertension candidate genes such as *WNK4*, *SCNN1B* and *SCNN1G* in hypertensive patients [18,19]. Such studies support the present study in the scenario that rare mutations collectively contribute to hypertension.

It has been reported that mice lacking *Rgs2* show not only a hypertensive phenotype but also reduced T-cell proliferation and IL-2 production, which indicates an impaired antiviral immunity *in vivo* [32]. Interestingly, homozygous *Rgs2*-null mice also display increased anxiety responses and decreased male aggression in the absence of cognitive or motor deficits. RGS2 also controls synaptic development and basal electrical activity in hippocampal CA1 neurons. RGS2 is thus important for T-cell activation; synapse development in the hippocampus, and emotive behaviours. These phenotypes were observed only in homozygous *Rgs2*-null

mice and not in heterozygous mice, suggesting that the rare mutations identified in our study might not induce these phenotypes in humans in whom it is a heterozygous state.

The mechanisms by which the two common polymorphisms (1026T > A, 1891–1892delTC) might contribute to hypertension in only women are unknown. The association in men was not observed. This inconsistency might be derived from sex differences or a lack of statistical power because of the sample size. Regarding the sex differences, RGS proteins are important in regulating signaling cascades initiated by GPCR activation, including both angiotensin II and endothelin-1 receptors. It is thought that these vasoactive peptides acting on GPCR may have different regulating mechanisms by sex. It has been reported that there are sex differences in aldosterone production after angiotensin II administration [33], and oestrogen influences angiotensinogen production through its regulation of the promoter region [34]. Endothelin-1 also acts on vasculatures differently in males and females. The elevation in blood pressure after endothelin-1 administration is much higher in male rats than in female rats [35], because oestrogen may reduce endothelin-1-induced vasoconstriction [36]. Therefore, we suppose that the cause of sex differences in the relationship between RGS2 polymorphisms and hypertension may be via the influences of these vasoactive pressor peptides. However, as both 1026T > A and 1891–1892delTC are located in an intronic region, we speculate that these mutations may be mere genetic markers, and other functional polymorphisms may play more important roles in hypertension.

In previous studies, we performed an association study of other genes using the same Suita population. In the study of genetic analysis of 118 SNP of 22 candidate genes for hypertension, multiple logistic analyses indicated that 13 SNP (eight genes), six SNP (four genes) and 11 SNP (four genes) were associated with hypertension ($P < 0.05$) in the total, male, and female population, respectively [37]. One of the SNP was significantly associated with hypertension in women even after correction by the Bonferroni method (corrected $P = 0.0236$). The analyses of 118 SNP of 22 genes would thus be required for the correction of the Bonferroni method to identify the possible candidate genes for hypertension. In the present study, we genotyped only three SNP that are in tight linkage disequilibrium. Therefore, we did not perform correction for multiple testing.

In summary, we suggest that the rare mutations in *RGS2*, Q2L, M5V, R44H, and 1925–1926insT collectively contribute to the pathogenesis of hypertension. The association study also suggests that RGS2 is associated with hypertension in the Japanese general population. As association studies are not consistently reproducible as a result of false positives, false negatives, and problems with true variability in association between different populations [38], the association of *RGS2* polymorphisms with hypertension has to be re-examined in another population. Further functional analyses of RGS mutants are also necessary to clarify the functional defects caused by these genetic findings.

Acknowledgements

The authors would like to express their highest gratitude to Dr Soichiro Kitamura, President of the National Cardiovascular Center, for his support of the millennium genome project. They would also like to express their gratitude to Drs Otosaburo Hishikawa, Katsuyuki Kawanishi, Tadashi Fujikawa, and Toshifumi Mannami for their continuous support of the population survey in Suita city. The authors would like to thank the members of the Satsuki-Junyukai, and also all the staffs in the Division of Hypertension and Nephrology, and Preventive Cardiology for supporting medical examination and Y. Tokunaga for technical assistance.

References

1 Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. *Cell* 2001; **104**:545–556.
2 Takahashi N, Smithies O. Gene targeting approaches to analyzing hypertension. *J Am Soc Nephrol* 1999; **10**:1598–1605.
3 Asico LD, Ladines C, Fuchs S, Accili D, Carey RM, Semeraro C, *et al.* Disruption of the dopamine D_3 receptor gene produces renin-dependent hypertension. *J Clin Invest* 1998; **102**:493–498.
4 Berthiaume N, Yanagisawa M, Labonte J, D'Orleans-Juste P. Heterozygous knock-out of ET_B receptors induces BQ-123-sensitive hypertension in the mouse. *Hypertension* 2000; **36**:1002–1007.
5 Kim HS, Krege JH, Kluckman KD, Hagaman JR, Hodgin JB, Best CF, *et al.* Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci USA* 1995; **92**:2735–2739.
6 Niranjan V, Telemaque S, deWit D, Gerard RD, Yanagisawa M. Systemic hypertension induced by hepatic overexpression of human preproendothelin-1 in rats. *J Clin Invest* 1996; **98**:2364–2372.
7 Bengtsson K, Melander O, Orho-Melander M, Lindblad U, Ranstam J, Rastam L, Groop L. Polymorphism in the β_1-adrenergic receptor gene and hypertension. *Circulation* 2001; **104**:187–190.
8 Bray MS, Krushkal J, Li L, Ferrell R, Kardia S, Sing CF, *et al.* Positional genomic analysis identifies the β_2-adrenergic receptor gene as a susceptibility locus for human hypertension. *Circulation* 2000; **101**: 2877–2882.
9 Liolitsa D, Powell JF, Prince M, Lovestone S. Association study of the 5-HT_{2A} receptor gene polymorphism, T102C and essential hypertension. *J Hum Hypertens* 2001; **15**:335–339.
10 Sierra C, Coca A, Gomez-Angelats E, Poch E, Sobrino J, de la Sierra A. Renin–angiotensin system genetic polymorphisms and cerebral white matter lesions in essential hypertension. *Hypertension* 2002; **39**: 343–347.
11 De Vries L, Zheng B, Fischer T, Elenko E, Farquhar MG. The regulator of G protein signaling family. *Annu Rev Pharmacol Toxicol* 2000; **40**:235–271.
12 Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, *et al.* Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. *J Neurosci* 1998; **18**:7178–7188.
13 Sinnarajah S, Dessauer CW, Srikumar D, Chen J, Yuen J, Yilma S, *et al.* RGS2 regulates signal transduction in olfactory neurons by attenuating activation of adenylyl cyclase III. *Nature* 2001; **409**:1051–1055.
14 Wu HK, Heng HH, Shi XM, Forsdyke DR, Tsui LC, Mak TW, *et al.* Differential expression of a basic helix–loop–helix phosphoprotein gene, G0S8, in acute leukemia and localization to human chromosome 1q31. *Leukemia* 1995; **9**:1291–1298.
15 Mansfield TA, Simon DB, Farfel Z, Bia M, Tucci JR, Lebel M, *et al.* Multilocus linkage of familial hyperkalaemia and hypertension, pseudohypoaldosteronism type II, to chromosomes 1q31–42 and 17p11–q21. *Nat Genet* 1997; **16**:202–205.

16 Heximer SP, Knutsen RH, Sun XG, Kaltenbronn KM, Rhee MH, Peng N, *et al.* Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J Clin Invest* 2003; **111**:445–452.
17 Tang M, Wang G, Lu P, Karas RH, Aronovitz M, Heximer SP, *et al.* Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure. *Nat Med* 2003; **9**:1506–1512.
18 Kamide K, Tanaka C, Takiuchi S, Miwa Y, Yoshii M, Horio T, *et al.* Six missense mutations of the epithelial sodium channel β and γ subunits in Japanese hypertensives. *Hypertens Res* 2004; **27**:333–338.
19 Kamide K, Takiuchi S, Tanaka C, Miwa Y, Yoshii M, Horio T, *et al.* Three novel missense mutations of *WNK4*, a kinase mutated in inherited hypertension, in Japanese hypertensives: implication of clinical phenotypes. *Am J Hypertens* 2004; **17**:446–449.
20 Okuda T, Fujioka Y, Kamide K, Kawano Y, Goto Y, Yoshimasa Y, *et al.* Verification of 525 coding SNPs in 179 hypertension candidate genes in the Japanese population: identification of 159 SNPs in 93 genes. *J Hum Genet* 2002; **47**:387–394.
21 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* 1997; **28**:518–525.
22 Mannami T, Baba S, Ogata J. Potential of carotid enlargement as a useful indicator affected by high blood pressure in a large general population of a Japanese city: the Suita study. *Stroke* 2000; **31**:2958–2965.
23 Mannami T, Baba S, Ogata J. Strong and significant relationships between aggregation of major coronary risk factors and the acceleration of carotid atherosclerosis in the general population of a Japanese city: the Suita study. *Arch Intern Med* 2000; **160**:2297–2303.
24 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* 2003; **26**:301–306.
25 Watson N, Linder ME, Druey KM, Kehrl JH, Blumer KJ. RGS family members: GTPase-activating proteins for heterotrimeric G-protein α-subunits. *Nature* 1996; **383**:172–175.
26 Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, *et al.* Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. *J Neurosci* 1998; **18**:7178–7188.
27 Hepler JR, Berman DM, Gilman AG, Kozasa T. RGS4 and GAIP are GTPase-activating proteins for $G_{q\alpha}$ and block activation of phospholipase Cβ by γ-thio-GTP-$G_{q\alpha}$. *Proc Natl Acad Sci USA* 1997; **94**:428–432.
28 Tesmer JJ, Berman DM, Gilman AG, Sprang SR. Structure of RGS4 bound to AlF_4^--activated G_{i1}: stabilization of the transition state for GTP hydrolysis. *Cell* 1997; **89**:251–261.
29 Heximer SP, Lim H, Bernard JL, Blumer KJ. Mechanisms governing subcellular localization and function of human RGS2. *J Biol Chem* 2001; **276**:14195–14203.
30 Salim S, Sinnarajah S, Kehrl JH, Dessauer CW. Identification of RGS2 and type V adenylyl cyclase interaction sites. *J Biol Chem* 2003; **278**:15842–15849.
31 Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R, Hobbs HH. Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science* 2004; **305**:869–872.
32 Oliveira-Dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Whishaw IQ, *et al.* Regulation of T cell activation, anxiety, and male aggression by RGS2. *Proc Natl Acad Sci USA* 2001; **97**:12272–12277.
33 Giacche M, Vuagnat A, Hunt SC, Hopkins PN, Fisher ND, Azizi M, *et al.* Aldosterone stimulation by angiotensin II: influence of gender, plasma renin, and familial resemblance. *Hypertension* 2000; **35**:710–716.
34 Fischer M, Baessler A, Schunkert H. Renin angiotensin system and gender differences in the cardiovascular system. *Cardiovasc Res* 2002; **53**: 672–677.
35 Tatchum-Talom R, Martel C, Labrie C, Labrie F, Marette A. Gender differences in hemodynamic responses to endothelin-1. *J Cardiovasc Pharmacol* 2000; **36(Suppl. 1)**:S102–S104.
36 Jiang C, Sarrel PM, Poole-Wilson PA, Collins P. Acute effect of 17 beta-estradiol on rabbit coronary artery contractile responses to endothelin-1. *Am J Physiol* 1992; **263**:H271–H275.
37 Iwai N, Tago N, Yasui N, Kokubo Y, Inamoto N, Tomoike H, Shioji K. Genetic analysis of 22 candidate genes for hypertension in the Japanese population. *J Hypertens* 2004; **22**:1119–1126.
38 Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 2003; **33**: 177–182.

Circ J 2005; **69:** 138–142

Association Analysis Between Hypertension and *CYBA*, *CLCNKB*, and *KCNMB1* Functional Polymorphisms in the Japanese Population

— The Suita Study —

Yoshihiro Kokubo, MD; Naoharu Iwai, MD; Naomi Tago, BS; Nozomu Inamoto, MD; Akira Okayama, MD; Hideyuki Yamawaki, PhD; Hiroaki Naraba, PhD; Hitonobu Tomoike, MD

Background Reproducibility of results is important for the validity of genetic association studies. Recently, 3 functional polymorphisms, G(–930)A in *CYBA*, T481S in *CLCNKB*, and E65K in *KCNMB1*, were reported to be associated with blood pressure (BP) status and the aim of this study was to confirm those findings using a large cohort representing the general Japanese population.

Methods and Results The study population consisted of 3,652 subjects recruited from the Suita study as representive of the general population in Japan. The genotypes of the 3 polymorphisms were determined by the TaqMan method. Logistic analysis indicated that the *CYBA*/G(–930)A polymorphism was associated with hypertension in male subjects. In the male population, the odds ratio of the GG genotype over GA+AA was 1.27 (95% confidence interval 1.01–1.57, p=0.034). Moreover, residuals of systolic and diastolic BP values were significantly higher in subjects with the GG genotype than in those with the GA or AA genotype (p=0.0007). However, such significant effects of the genotype on BP status were not observed in the female population. The significance of the *CLCNKB*/T481S and *KCNMB1*/E65K polymorphisms were not replicated in the present study.

Conclusion The significance of the G(–930)A polymorphism of *CYBA* was confirmed in the present study with adequate statistical power, which strengthens the hypothesis that this polymorphism is important in the pathogenesis of hypertension and confers susceptibility. (*Circ J* 2005; **69:** 138–142)

Key Words: Epidemiology; Genetic association study; Hypertension

Essential hypertension (HT) is a multifactorial disorder influenced by both genetic and environmental factors. Over the past few years, a large number of genetic polymorphisms of candidate genes have been tested for their association with HT, but with controversial results, probably because of inadequate sample size, ethnic differences, and/or population stratification. The practical implications of an association study strongly depend on the reproducibility of the findings[1]

Recently, 3 specific functional polymorphisms have been reported to be associated with HT: the G(–930)A polymorphism of *CYBA* ($p22^{phox}$)[2] the E65K polymorphism of *KCNMB1*[3] and the T481S polymorphism of *CLCNKB*[4,5]

CYBA ($p22^{phox}$) is a major component of NAD(P)H oxidase, and the NAD(P)H oxidase system is considered to be the most important source of superoxide anion in vascular tissues[6,7] The superoxide anion has been suspected of involvement in the pathogenesis of HT through the inactivation of NO produced by NO synthetase (*NOS3*) in the vascular endothelium. Moreno et al reported that the G allele of *CYBA* had higher promoter activity and is associated with HT[2]

(Received October 22, 2004; revised manuscript received November 19, 2004; accepted December 1, 2004)

National Cardiovascular Center, Suita, Osaka, Japan
Mailing address: Naoharu Iwai, MD, Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail: iwai@ri.ncvc.go.jp

Blood pressure (BP) depends on the resistance of resistant vessels, and a key element of arterial tone is the large-conductance Ca^{2+} and voltage-dependent K^+ (BK) channel. The BK channel is formed by an ion-conducting α subunit and a regulatory β_1 subunit (*KCNMB1*). An activating gain-of-function mutation, the K65 allele, is reported to be associated with a low prevalence of diastolic HT[3]

The chloride channel ClC-Kb is expressed in the distal nephron and is known to be responsible for classic Bartter syndrome. The S481 allele has been reported to confer a strong gain-of-function effect that leads to enhanced NaCl re-absorption, and as expected, susceptibility to HT, in a Caucasian population[5]

All 3 polymorphisms have functional significance and are thus intriguing candidate genes. However, the sample sizes of the studies to date have been relatively small, or the descriptions of the epidemiological aspects rather weak. The purpose of the present study was to replicate findings of the previous association studies in a large epidemiological cohort representing the general population in Japan and to evaluate the possible importance of these polymorphisms.

Methods

Study Population

The selection criteria and design of the Suita Study have been previously described[8–10] The present study was

Table 1 TaqMan Probes and Primers for *CYBA*, *CLCNKB*, and *KCNMB1*

	Probe		Primer	
	VIC	FAM	F	R
CYBA G(−930)A	CCAGCATTACTGCCTC	CAGCATTGCTGCCTC	GCCCCGGTGGCCAT	GAAACAGAAAAACGGCGGAG
CLCNKB T481S	TGACCCACACCATCTC	TGACCCACTCCATCTC	TGCAGCCTTCTCAGGGGC	ACCTCGAAGGCCAGCAGC
KCNMB1 K65E	CTTCAGCTTCTCCTGGT	TTCAGCTCCTCCTGGT	CGGCAGCTGACACGTTGA	CCAAGTGCCACCTGATTGAGA

Table 2 Characteristics of the Study Population

	Male	Female	p value	Hypertension	Normotension	p value
N	1,706	1,946		1,522	2,130	
M/F				776/746	930/1,200	<0.0001
Age (years)	66.12 (0.27)	63.47 (0.25)	<0.0001	68.81 (0.28)	61.77 (0.23)	<0.0001
Body mass index	23.32 (0.08)	22.38 (0.07)	<0.0001	23.56 (0.08)	22.29 (0.07)	<0.0001
HTN (%)	45.49	38.34	<0.0001			
AHT (%)	28.19	22.61	<0.0001	60.5	0	<0.0001
CVA (%)	4.1	1.85	<0.0001	5.12	1.31	<0.0001
MI (%)	2.29	0.57	<0.0001	2.23	0.75	0.0001
Drinking (%)	66.88	27.34	<0.0001	48.88	43.62	0.0070
Smoking (%)	30	5.96	<0.0001	14.19	19.34	<0.0001

Data are mean (standard error). Differences between the 2 groups (male vs female, hypertensives vs normotensives) were calculated by t-test or χ^2 analysis.
HTN, hypertension; AHT, antihypertensive medication; CVA, cerebrovascular accident; MI, myocardial infarction; drinking, alcohol drinking habbit; smoking, cigarette smoking habit.

Table 3 Genotype Distribution of *CYBA*/G(−930)A, *CLCNKB*/T481S, and *KCNMB1*/E65K in Hypertensive Subjects

	Major	Hetero	Minor	p value
CYBA				
G>A				
M	258/523 (49.3)	378/836 (45.2)	139/344 (40.4)	0.0344 (0.0393)
F	223/573 (38.9)	371/963 (38.5)	146/401 (36.4)	0.6990 (0.7189)
T	481/1,096 (43.9)	749/1,799 (41.6)	285/745 (38.3)	0.0548 (0.0886)
CLCNKB				
T>S				
M	747/1,644 (45.4)	26/54 (48.1)		0.6943 (0.6076)
F	720/1,879 (38.3)	20/58 (34.5)		0.5512 (0.3490)
T	1,467/3,523 (41.6)	46/112 (41.1)		0.9042 (0.7529)
KCNMB1				
E>K				
M	603/1,332 (45.3)	162/349 (46.4)	10/20 (50.0)	0.8577 (0.8967)
F	582/1,560 (37.3)	147/348 (42.2)	11/24 (45.8)	0.1757 (0.2646)
T	1,185/2,892 (41.0)	309/697 (44.3)	21/44 (47.7)	0.1966 (0.3478)

M, male subjects; F, female subjects; T, total (male+female) subjects. Major, Hetero, and Minor indicate major genotype, heterozygous genotype, and minor genotype, respectively.
The [number of hypertensive subjects/number of normotensive+hypertensive subjects] and (% of hypertensive subjects) are indicated. p values are calculated by χ^2 analyses using genotype as an independent variable; values in parentheses are p values obtained by multiple logistic analysis with the genotype (GG, GA and AA) as independent variable and age and BMI as covariates.

approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center. The genotypes were determined in 3,652 subjects recruited from the Suita Study between April 2002 to February 2004. All subjects provided written informed consent.

DNA Studies

The polymorphisms were determined by the TaqMan system as described previously[11] The primers and probes are summarized in Table 1.

Statistical Analysis

Values are expressed as the mean±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute, Inc, Cary, NC, USA). Multiple logistic analyses (presence of HT) were performed with age and body mass index (BMI) as covariates. Subjects were categorized as hypertensive (HTN) when they had a systolic BP (SBP) of 140 mmHg or higher and/or a diastolic BP (DBP) of 90 mmHg or higher. Subjects who were currently taking antihypertensive medication were also categorized as HTN. The effects of the polymorphisms on BP values were evaluated by excluding subjects who were taking antihypertensive medications, since HTN that has excellent BP control by medication may exhibit normal values. Residuals of the BP values were calculated by adjusting for age and BMI (Residuals represent the difference between the actual BP value for each observation and the value predicted on the basis of age and BMI). Differences among the groups were calculated by one-way AVOVA. The differences in frequencies were calculated by χ^2 analysis. The

Table 4 Blood Pressure Values According to the *CYBA*/G(−930)A, *CLCNKB*/T481S, and *KCNMB1*/E65K Genotype

	Major	*Hetero*	*Minor*	*p value*
Residuals of SBP				
CYBA				
G>A				
M	2.4 (0.9) [380]	−0.4 (0.7) [593]	−2.7 (1.1) [250]	0.0007
F	0.3 (0.8) [455]	0.0 (0.6) [728]	−0.4 (0.9) [319]	0.8440
T	1.3 (0.6) [835]	−0.3 (0.5) [1,321]	−1.3 (0.7) [569]	0.0108
CLCNKB				
T>S				
M	0.0 (0.5) [1,183]	−0.1 (2.8) [37]		0.9454
F	0.0 (0.4) [1,453]	−0.2 (2.4) [49]		0.9228
T	0.0 (0.3) [2,636]	0.0 (1.8) [86]		0.9732
KCNMB 1				
E>K				
M	−0.2 (0.5) [950]	1.1 (1.1) [256]	−0.6 (4.5) [14]	0.4955
F	−0.3 (0.5) [1,216]	1.4 (1.0) [265]	0.0 (4.2) [16]	0.3677
T	−0.3 (0.4) [2,166]	1.3 (0.7) [521]	−0.4 (3.1) [30]	0.1817
Residuals of DBP				
CYBA				
G>A				
M	1.0 (0.5) [380]	0.0 (0.4) [593]	−1.5 (0.6) [250]	0.0072
F	0.4 (0.4) [455]	−0.1 (0.3) [728]	−0.2 (0.5) [319]	0.6300
T	0.7 (0.3) [835]	−0.1 (0.3) [1,321]	−0.7 (0.4) [569]	0.0170
CLCNKB				
T>S				
M	0.0 (0.5) [1,183]	−0.1 (2.8) [37]		0.9454
F	0.0 (0.2) [1,453]	−0.4 (1.3) [49]		0.7633
T	0.0 (0.2) [2,636]	−0.3 (1.0) [86]		0.7624
KCNMB 1				
E>K				
M	0.1 (0.3) [950]	−0.3 (0.6) [256]	2.0 (2.6) [14]	0.6760
F	0.0 (0.3) [1,216]	−0.2 (0.6) [265]	1.1 (2.3) [16]	0.8420
T	0.0 (0.2) [2,166]	−0.1 (0.4) [521]	1.4 (1.7) [30]	0.6885
Hypertension				
CYBA				
G>A				
M	115/265	135/458	45/205	0.0014
F	105/350	136/592	64/255	0.2681
T	220/615	271/1,050	109/460	0.0014
CLCNKB				
T>S				
M	286/897	9/28		0.9206
F	294/1,159	11/38		0.8566
T	580/2,056	20/66		0.9912
KCNMB 1				
E>K				
M	222/726	68/186	4/10	0.4581
F	234/973	63/200	3/13	0.2889
T	456/1,699	131/386	7/23	0.1359

DBP, diastolic blood pressure; SBP, systolic blood pressure. Other abbreviations as in Table 3.
Residuals of systolic and diastolic blood pressure values were calculated by adjusting for age and BMI. Values (mmHg) are shown as mean (SEM). [Number]indicates the number of subjects in each group.
Subjects who were taking antihypertensive medication were excluded from this analysis. Prevalences of hypertension according to the genotypes in this study population (exclusing those with antihypertensive medication) are also shown at the bottom. p values are calculated by adjusting for age and BMI.

sample power was calculated using the Sample Power statistical package (SPSS Inc, Chicago, IL, USA).

Results

The characteristics of the study population are given in Table 2. The effects of the 3 polymorphisms on HT and BP are shown in Tables 3 and 4.

CYBA/G(−930)A

Multiple logistic analysis with age and BMI as covariates indicated that the GG (vs GA+AA) genotype was associated with HT, with an odds ratio of 1.27 (95% confidence interval 1.01–1.57, p=0.0340), in the male population. The genotype frequencies of the GG, GA, and AA genotypes were 0.307, 0.491, and 0.202 (Table 3). Based on these frequencies and the sample size (male n=1,703), the sample power of this statistic was calculated to be 0.75 (α=0.05, two-tailed). In this sample power calculation, subjects with the GA or AA genotype were categorized into one group. In males, residuals of the SBP and DBP values were also significantly higher in subjects with the GG genotype than in those with the GA or AA genotype (Table 4). The difference between the residuals of SBP for the GG and AA genotypes was 5.1 mmHg. Consistency between the analysis of the categorical data and the analysis of the numerical

data might strengthen the hypothesis that the *CYBA* promoter variant contributes to HT in men. Such significant effects of the genotype on BP status were not observed in the female population, although a similar non-significant trend was observed.

CLCNKB/T481S

We did not find any significant association between the *CLCNKB*/T481S polymorphism and HT, SBP, or DBP in male or female subjects (Tables 3,4).

KCNMB1/E65K

We did not find any significant association between the *KCNMB1*/E65K polymorphism and and HT, SBP, or DBP in male or female subjects (Tables 3,4). Fernandez-Fernandez et al reported that the genotype frequency (KK+KE) decreased with increasing DBP values;[3] so on that basis we categorized subjects without HT medication into 4 groups based on the DBP values (Group-I: <79 mmHg; 80≤ Group-II <90 mmHg; 90≤ Group-III <99 mmHg; Group-IV: ≥100 mmHg). The respective genotype (KK+KE) frequencies were 20.4% (n=1,746), 20.3% (n=715), 19.4% (n=201), and 17.8% (n=39). No significant difference in the genotype frequency was observed among the 4 groups.

Discussion

Over the past decade, many genetic association studies have been performed with inconsistent results, and we are now recognizing that the odds of common HT alleles are less than expected![1,10] Thus, any single study that considers just a few thousand subjects may not be large enough to reach concrete conclusions and should be viewed as providing tentative results only.

In the present study, the genotype frequency of the heterozygote of the T481S polymorphism was just 0.03, and none of the subjects was homozygous for the 481S allele. This heterozygous genotype was expected to be associated with HT through enhanced sodium chloride reabsorption, but given its small frequency, odds of more than 1.76 would be required (α=0.05, two-tailed) to observe an association with a sample size of 3,652 subjects. Thus, a practical implication of the present study is that the odds of the heterozygous genotype of the T481S polymorphism, if any, should be less than 1.76. It is possible that the effects of this activating polymorphism could be more clearly observed under salt-loading conditions or in subjects homozygous for the mutation. An even larger study population with information on salt intake might be required to evaluate the significance of this polymorphism.

That situation is also true for the E65K polymorphism of *KCNMB1*. The K65 allele, an activating mutation of *KCNBM1*, was expected to be associated with a lower prevalence of HT. The frequency of the EK+KK genotype was 0.20, and therefore odds of less than 0.79 would be required (α=0.05, two-tailed) to observe an association with a sample size of 3,652. Thus, the present study indicated that the contribution of the E65K polymorphism, if any, is very slight, with an odds ratio of more than 0.79.

Our failure to replicate the possible involvement of the *CLCNKB*/T481S and *KCNMB1*/E65K polymorphisms in BP regulation might be ascribed to ethnic differences, which include not only genetic but also environmental differences. A genetic variation may be differentially expressed under different conditions.

The G(−930) allele of *CYBA* has been reported to have higher promoter activity, and may be associated with higher production of superoxide anion in vascular tissues.[2] The superoxide anion has suspected involvement in the pathogenesis of HT by inactivating NO produced by NO synthetase (*NOS3*) in the vascular endothelium.[6,7] The discrepancy between males and females in terms of the effects of the G(−930)A polymorphism on BP might be interpreted from the perspective of estrogen. Estrogen stimulates the production of NO in vascular tissues,[12–14] and the effect of NO produced by estrogen on BP control was observed in mice deficient in the estrogen β-receptor![15] It is possible that the NO-inactivating-property of the G(−930) allele may be overcome in females because of higher levels of NO produced in the vascular tissues by estrogen.

Gender differences in superoxide generation in microvessels have been reported in the spontaneously hypertensive rat, and have been attributed to AT-1-dependent overexpression of the components of NAD(P)H oxidase![16] It is also possible that sexual dimorphism in the effects of the G(−930)A polymorphism on BP may be related to different expression of the CYBA protein.

The present study results indicate that, of the 3 polymorphisms investigated, the G(−930)A polymorphism of *CYBA* seems to be the most promising genetic variant conferring susceptibility to HT in males. From a clinical viewpoint, it might be interesting to investigate whether HTN with the GG genotype are more responsive to nitrate derivatives, bearing in mind our earlier concern about the size of any particular study in relation to its results.

Acknowledgments

We express our highest gratitude to the following people for their continuous support of our population survey: Dr Otosaburo Hishikawa, President; Dr Katsuyuki Kawanishu, Committee in Chief for the city health check-up service; other members of the Suita City Medical Association; and Mr Shigeru Kobayashi, Director of the City Health Center. We also express our great thanks to the members of our attendants' society (Satsuki-Junyu-kai) for their cooperation and assistance with our survey of risk factors and preventive activity on cardiovascular diseases. We also thank Professor Soichiro Kitamura, President of the National Cardiovascular Center, for considering our research work.

This study was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan (MPJ-3), and by grant-in-aid from the Salt Science Research Foundation, No.04C5.

References

1. Harrap SB. Private genes, public health. *Lancet* 1997; **349:** 1338–1339.
2. Moreno MU, Jose GS, Orbe J, Paramo JA, Beloqui O, Diez J, et al. Preliminary characterisation of the promoter of the human p22^{phox} gene: Identification of a new polymorphism associated with hypertension. *FEBS Lett* 2003; **542:** 27–31.
3. Fernandez-Fernandez JM, Tomas M, Vazquez E, Orio P, Latorre R, Senti M, et al. Gain-of-function mutation in the KCNMB1 potassium channel subunit is associated with low prevalence of diastolic hypertension. *J Clin Invest* 2004; **113:** 1032–1039.
4. Jeck N, Waldegger P, Doroszewicz J, Seyberth H, Waldegger S. A common sequence variation of the CLCNKB gene strongly activates CIC-Kb chloride channel activity. *Kidney Int* 2004; **65:** 190–197.
5. Jeck N, Waldegger S, Lampert A, Boehmer C, Waldegger P, Lang PA, et al. Activating mutation of the renal epithelial chloride channel CIC-Kb predisposing to hypertension. *Hypertension* 2004; **43:** 1175–1181.
6. Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 1986; **320:** 454–456.
7. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, et al. Mechanisms of increased vascular superoxide production in human diabetes mellitus: Role of NAD(P)H oxidase and endothelial

nitric oxide synthase. *Circulation* 2002; **105:** 1656–1662.
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* 1997; **28:** 518–525.
9. Iwai N, Tago N, Yasui N, Kokubo Y, Inamoto N, Tomoike H, et al. Genetic analysis of 22 candidate genes for hypertension in the Japanese population. *J Hypertens* 2004; **22:** 1119–1126.
10. Iwai N, Katsuya T, Mannami T, Higaki J, Ogihara T, Kokame K, et al. Association between SAH, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. *Circulation* 2002; **105:** 41–47.
11. Shioji K, Kokubo Y, Mannami T, Inamoto N, Morisaki H, Mino Y, et al. Association between hypertension and the alpha-adducin, beta1-adrenoreceptor, and G-protein beta3 subunit genes in the Japanese population: The Suita study. *Hypertens Res* 2004; **27:** 31–37.
12. Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci USA* 1994; **91:** 5212–5216.
13. Goetz RM, Morano I, Calovini T, Studer R, Holtz J. Increased expression of endothelial constitutive nitric oxide synthase in rat aorta during pregnancy. *Biochem Biophys Res Commun* 1994; **205:** 905–910.
14. Huang A, Sun D, Koller A, Kaley G. Gender difference in flow-induced dilation and regulation of shear stress: Role of estrogen and nitric oxide. *Am J Physiol Regul Integr Comp Physiol* 1998; **275:** R1571–R1577.
15. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, et al. Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. *Science* 2002; **295:** 505–508.
16. Dantas APV, Franco MDCP, Silva-Antonialli MM, Tostes RCA, Fortes ZB, Nigro D, et al. Gender differences in superoxide generation in microvessels of hypertensive rats: Role of NAD(P)H-oxidase. *Cardiovasc Res* 2004; **61:** 22–29.

Circ J 2005; **69:** 131–137

Effects of the Endothelin Receptor Antagonist Bosentan on Hemodynamics, Symptoms and Functional Capacity in Japanese Patients With Severe Pulmonary Hypertension

Shigetake Sasayama, MD; Takeyoshi Kunieda, MD*; Hitonobu Tomoike, MD**; Masunori Matsuzaki, MD†; Kunio Shirato, MD††; Takayuki Kuriyama, MD‡; Tohru Izumi, MD‡‡; Hideki Origasa, PhD¶; Paul LM van Giersbergen, PhD¶¶; Jasper Dingemanse, PhD¶¶; Satoshi Tanaka, MD¶¶

Background Endothelin (ET)-1 has a pathogenic role in pulmonary arterial hypertension (PAH). Recent clinical studies carried out in Western populations showed that blockade of the ET receptors by bosentan improves pulmonary hemodynamics and exercise capacity. In the present study, the efficacy of bosentan was assessed in Japanese patients with PAH.

Method and Results Because the pharmacokinetics of bosentan and its metabolites are similar in Japanese and Caucasian subjects, the same dose of bosentan, 125 mg twice daily, was administered in the Japanese open-label clinical trial. In 18 patients, mean pulmonary arterial pressure decreased from 52.4±13.8 to 46.8±13.8 mmHg ($p=0.003$) and cardiac index increased from 2.20±0.74 to 2.61±0.72 $L \cdot min^{-1} \cdot m^{-2}$ ($p=0.002$). The 6-min walking distance increased from 410±89.5 to 494±86.0 m ($p<0.0001$). The dyspnea index (Borg scale) decreased from 3.2±2.4 to 2.2±1.7 ($p=0.02$). The specific activity scale (SAS) gradually increased throughout the study period from 2.9±0.8 to 4.6±1.9 METs ($p=0.0005$). WHO Class improved in 10 patients.

Conclusion Bosentan was well tolerated and improved the hemodynamics, symptoms, exercise capacity, and quality of life of Japanese patients with PAH. Thus, bosentan can be a valuable therapeutic option in Japanese patients. (*Circ J* 2005; **69:** 131–137)

Key Words: Bosentan; Endothelin receptor antagonist; Pulmonary arterial hypertension; Quality of life

Pulmonary arterial hypertension (PAH) is a rare and debilitating disease, characterized by an increase in pulmonary vascular resistance that ultimately leads to right heart failure and death[1] When a definite cause can not be demonstrated, the condition is termed primary pulmonary hypertension (PPH), which predominantly affects women most commonly in their third decade of life[2] No ethnic predisposition is apparent in the National Institutes of Health registry, and the proportions by ethnic group parallel those in the general population[2] Similar pulmonary vascular lesions are produced by many illnesses such as scleroderma, human immunodeficiency virus infection, liver disease or the use of certain anorectic drugs, and these are now classified as types of PAH[3] A limited number of innovative strategies for the treatment of PAH have been developed over the past decades, but their effectiveness is largely limited by their nonselectivity for the pulmonary vasculature and significant drawbacks have been reported[5]

Recently, it was shown that PAH is associated with increased concentrations of endothelin (ET)-1, a potent vasoconstrictor, in plasma and the lungs[6,7] suggesting that inhibition of ET receptors is a potential therapeutic alternative for this life-threatening disorder. In fact, studies with Caucasian PAH patients have demonstrated significant clinical benefits of bosentan, a dual ET receptor antagonist[8–10] In the present study, the effects of bosentan on cardiopulmonary hemodynamics, symptoms and functional capacity were assessed, as well as the 6-min walk test and the specific activity scale (SAS), in Japanese patients with PAH.

The pharmacokinetics of bosentan are dose-proportional up to a dose of 500 mg and in Caucasians, the absolute bioavailability of bosentan is 50%, being mainly excreted via the bile in the form of metabolites[11,12] However, ethnic differences in the pharmacokinetics of many drugs have been demonstrated[13] Therefore, prior to the start of the clinical trial, the multiple-dose pharmacokinetics of bosentan were compared in Caucasian and Japanese subjects.

Methods

Comparative Study of the Ethnic Differences in the Pharmacokinetics of Bosentan

This part of the study was performed at FOCUS GmbH

(Received August 18, 2004; revised manuscript received November 1, 2004; accepted November 8, 2004)

*Hamamatsu Rosai Hospital, Hamamatsu, *Ise Keio Hospital, Ise, **National Cardiovasular Center, Suita, †Yamaguchi University Graduate School of Medicine, Ube, ††Tohoku University Graduate School of Medicine, Sendai, ‡Chiba University Graduate School of Medicine, Chiba, ‡‡Kitasato University School of Medicine, Sagamihara, ¶Toyama Medical and Pharmaceutical University, Toyama, Japan and ¶¶Actelion Pharmaceuticals Ltd, Allschwil, Switzerland*

Mailing address: Shigetake Sasayama, MD, Hamamatsu Rosai Hospital, 25 Shogen-cho, Hamamatsu 430-8525, Japan

(Neuss, Germany). The protocol was approved by the independent Ethics Committee of the "Aerztekammer Nordrhein" (Düsseldorf, Germany). All subjects gave written informed consent before any screening procedures were performed. Six male and 7 female healthy Caucasians (age 23–49 years) and 6 male and 7 female Japanese subjects (age 21–45 years) were assigned to treatment with 125 mg b.i.d. of bosentan for 7.5 days. Although the pharmacokinetics of bosentan are not influenced by food,[11,12] the meals were standardized and Japanese subjects received typical Japanese food and European food was served to the Caucasian subjects throughout the study period.

Blood samples of 4 ml were obtained immediately before drug administration in the morning of days 2–8 and at several time points (every hour for the first 6 h, every 2 h for the subsequent 10 h and finally after 24 h) after drug administration on day 8. Plasma was separated and stored at −20°C pending analysis. The concentration of bosentan and its active metabolite, Ro 48-5033 were determined by a liquid chromatography method with tandem mass spectrometry detection.[14] The limit of quantification was 1.0 ng/ml for bosentan and 2.0 ng/l for Ro48-5033.

The pharmacokinetic evaluation for bosentan and Ro48-5033 used model independent methods.[15] The peak plasma concentration (C_{max}) and the time to C_{max} (t_{max}) were read directly from the concentration–time data. The area under the plasma concentration–time curve (AUC) was estimated by the linear trapezoidal rule and log-linear regression analysis of the terminal phase. Pharmacokinetic parameters were analyzed descriptively, calculating the geometric mean and 95% confidence intervals or for t_{max}, the median and range.

The study was powered to detect with 90% power a difference of 50% in AUC_τ between the 2 ethnic groups. Differences between Caucasian and Japanese subjects for the bosentan and metabolite pharmacokinetic parameters were explored using the 2-sample t-test on logarithmically transformed C_{max}, AUC_τ, and $t_{1/2}$ values, and the 2-sample Wilcoxon test for t_{max}.

Clinical Study of the Effects of Bosentan in Japanese Patients With PAH

Japanese patients aged over 20 years were eligible for enrollment in the study if they (1) had symptomatic, severe PPH or PAH because of connective-tissue disease (scleroderma or systemic lupus erythematosus (SLE)), (2) were in functional classes III–IV according to the 1998 World Health Organization (WHO) classification despite conventional therapy, (3) met the following hemodynamic criteria within 2 months of enrollment: mean pulmonary arterial pressure (mPAP) >25 mmHg at rest, pulmonary capillary wedge pressure (PCWP) <15 mmHg, and pulmonary vascular resistance (PVR) >240 dyn·s/cm^5, (4) had a baseline 6-min walk test between 150 and 500 m. Patients were excluded if they were pregnant, had hypotension (systolic blood pressure <100 mmHg), hypokalemia or other significant systemic disease. The institutional ethics review committees approved the protocol and written informed consent was obtained from all patients.

At baseline, within 2 months prior to the start of treatment, hemodynamic measurements were performed with a Swan-Ganz catheter while patients were recumbent. Cardiac output (CO) was obtained by the thermodilution method using the mean of 3 measurements. The cardiac index (CI) was derived by normalization of CO with the body surface area (BSA) (CI=CO/BSA). PVR was calculated from the transpulmonary gradient and CO (PVR=[mPAP−PCWP]/CO). The patients' symptoms were evaluated by the Borg dyspnea index (a measure of perceived breathlessness on a scale of 0–10, with higher values indicating more severe dyspnea)[16] and the WHO functional class for pulmonary hypertension. Efficacy of treatment was also assessed by the 6-min walk test and the specific activity scale (SAS).[17] To determine the SAS, patients were asked to specify the extent of physical activity they could perform without symptomatic limitation. Summarizing these data, the patient was categorized by the metabolic costs expended with the most strenuous possible activity.

After the baseline assessments, bosentan (Tracleer, Actelion, Allschwil, Switzerland) was started at a dose of 62.5 mg once daily for the first week, then 62.5 mg twice daily for the next 3 weeks, and finally 125 mg twice daily for the subsequent 8 weeks. Hemodynamic measurements were performed after the 12 weeks of treatment. Symptoms, physical examinations, electrocardiogram, 6-min walk test, WHO classification, and SAS were assessed every 4 weeks. Safety was assessed on the basis of recorded adverse events, clinical laboratory parameters, vital signs, and electrocardiography.

Statistical Analysis

The PVR as the primary efficacy parameter, and other hemodynamic values at week 12 were compared with the baseline on a per protocol population basis by using the signed-ranks test as primary analysis. A significant change was defined as $p<0.05$ (two-tailed). In a patient in whom bosentan treatment was terminated because of worsening of the disease, the hemodynamic data obtained at the last observation were used for analysis. If data were not available, the imputation rule of using the worst data (pre-treatment value in this case) was used. If the data at 12 weeks were not available because of termination of the treatment for other reasons, the last data between 8 and 12 weeks were adopted for analysis. The missing values for other measurements were excluded from the analysis. To confirm the robustness of the results, sensitive analysis based on the ITT (intention to treat) was used.

Results

Comparative Study of the Ethnic Differences in the Pharmacokinetics of Bosentan

Twenty-six subjects participated in the study and 24 completed the entire study in accordance with the protocol. Two subjects prematurely withdrew because of adverse events: myalgia of moderate intensity in 1 female Caucasian and a first-degree atrioventricular block in 1 Japanese female subject. Therefore, 26 subjects were evaluated for safety and 24 for pharmacokinetics.

Steady-state concentrations of bosentan were attained after 5–6 days of administration in both ethnic groups (data not shown). The mean plasma concentration–time curves and pharmacokinetic parameters of bosentan and its metabolite, Ro48-5033, are presented in Fig 1 and Table 1. The 2-sample t-test did not yield any statistically significant differences between the 2 ethnic groups.

Of the 47 adverse events that occurred during the study, 19 were reported by Caucasian and 28 by Japanese subjects. Headache of mild to moderate intensity was the most frequently reported adverse event in both ethnic

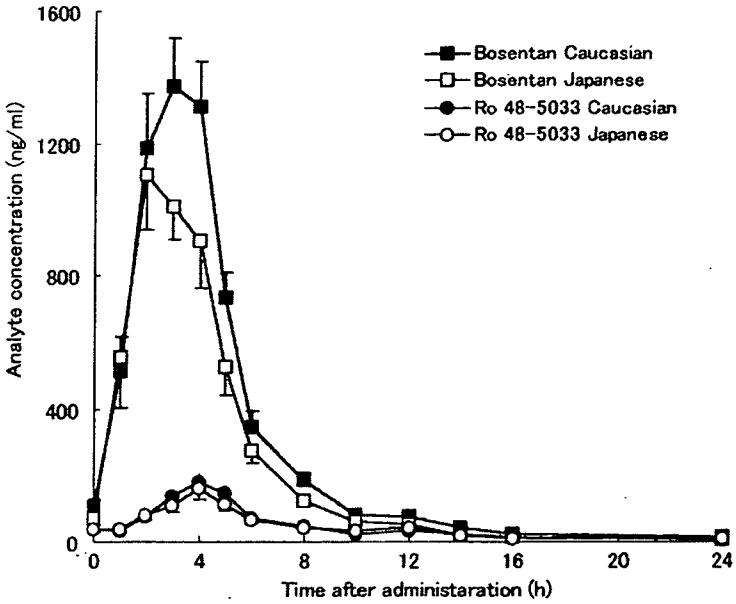


Fig 1. Mean plasma concentration–time curves of bosentan and its metabolite, Ro48-5033 in 12 healthy Caucasian and Japanese subjects after administration of 125 mg of bosentan. Data are presented as arithmetic means ± SEM.

Table 1 Pharmacokinetic Parameters of Bosentan and Its Metabolite in Caucasian and Japanese Subjects After Administration of 125 mg of Bosentan

Group	*C_{max} (ng/ml)*	*t_{max} (h)*	*AUC (ng·h/ml)*	*$t_{1/2}$*
Bosentan				
Caucasian	*1,434*	*3.5*	*6,046*	*7*
	(1,137, 1,808)	*(2.0, 4.0)*	*(4,997, 7,311)*	*(5.3, 9.3)*
Japanese	*1,212*	*3*	*4,640*	*5.6*
	(940, 1,564)	*(1.0, 4.0)*	*(3,641, 5,914)*	*(4.6, 6.9)*
Ro 48-5033				
Caucasian	*175*	*4*	*859*	*10.6*
	(138, 221)	*(3.0, 5.0)*	*(3,641, 5,914)*	*(4.6, 6.9)*
Japanese	*136*	*4*	*721*	*9.6*
	(92, 201)	*(3.9, 5.0)*	*(532, 977)*	*(7.7, 11.8)*

Data are expressed as geometric mean (95% confidence limits).
AUC, area under curve; C_{max}, peak plasma concentration; t_{max}, time to C_{max}.

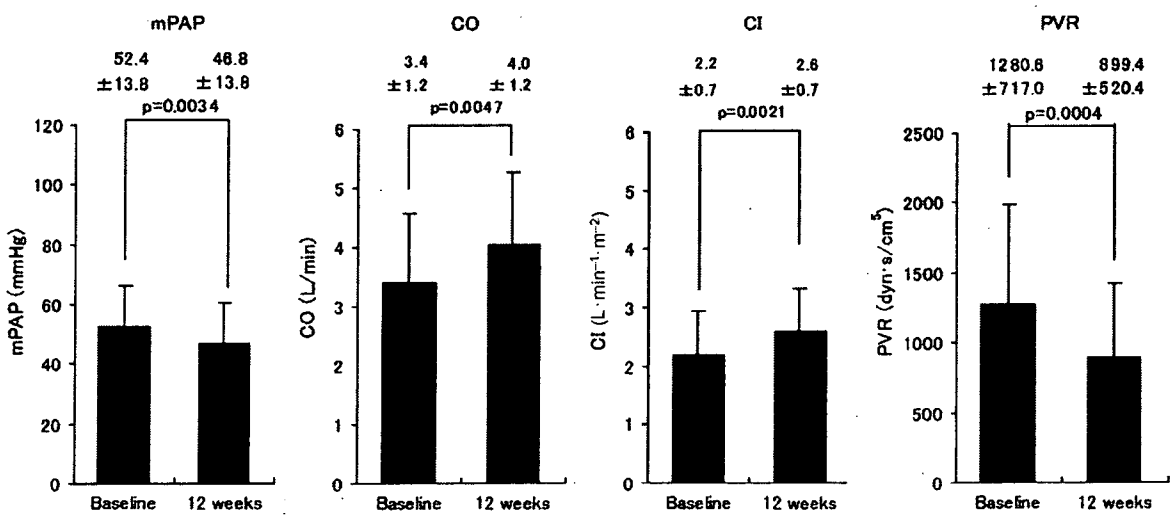


Fig 2. Effect of bosentan on hemodynamic parameters from baseline to week 12 (mean ± SEM). mPAP, mean pulmonary arterial pressure; CO, cardiac output; CI, cardiac index; PVR, pulmonary vascular resistance.

Table 2 Changes in the Hemodynamic Parameters After 12-Week Treatment Program With Bosentan 125 mg b.i.d. in 18 Patients With Severe Pulmonary Hypertension

Hemodynamic parameters	*Baseline*	*Week 12*	*p value*	*ITT**
Systolic pulmonary arterial pressure (mmHg)	*85.9±23.6*	*76.7±23.7*	*0.0106*	*0.0074*
Diastolic pulmonary arterial pressure (mmHg)	*33.1±8.6*	*28.3±9.1*	*0.0147*	*0.0182*
Mean pulmonary arterial pressure (mmHg)	*52.4±13.8*	*46.8±13.8*	*0.0034*	*0.0030*
Pulmonary capillary wedge pressure (mmHg)	*6.3±2.7*	*7.8±3.4*	*0.0309*	*0.0297*
Cardiac output (L/min)	*3.39±1.19*	*4.02±1.22*	*0.0047*	*0.0192*
Cardiac index ($L \cdot min^{-1} \cdot m^{-2}$)	*2.20±0.74*	*2.61±0.72*	*0.0021*	*0.0135*
Pulmonary vascular resistance ($dyn \cdot s/cm^5$)	*1,281±717*	*899±520*	*0.0004*	*0.0003*
Right arterial pressure (mmHg)	*4.9±4.0*	*5.4±3.7*	*0.3134*	*0.3510*

**Sensitive analysis by intension-to-treat.*

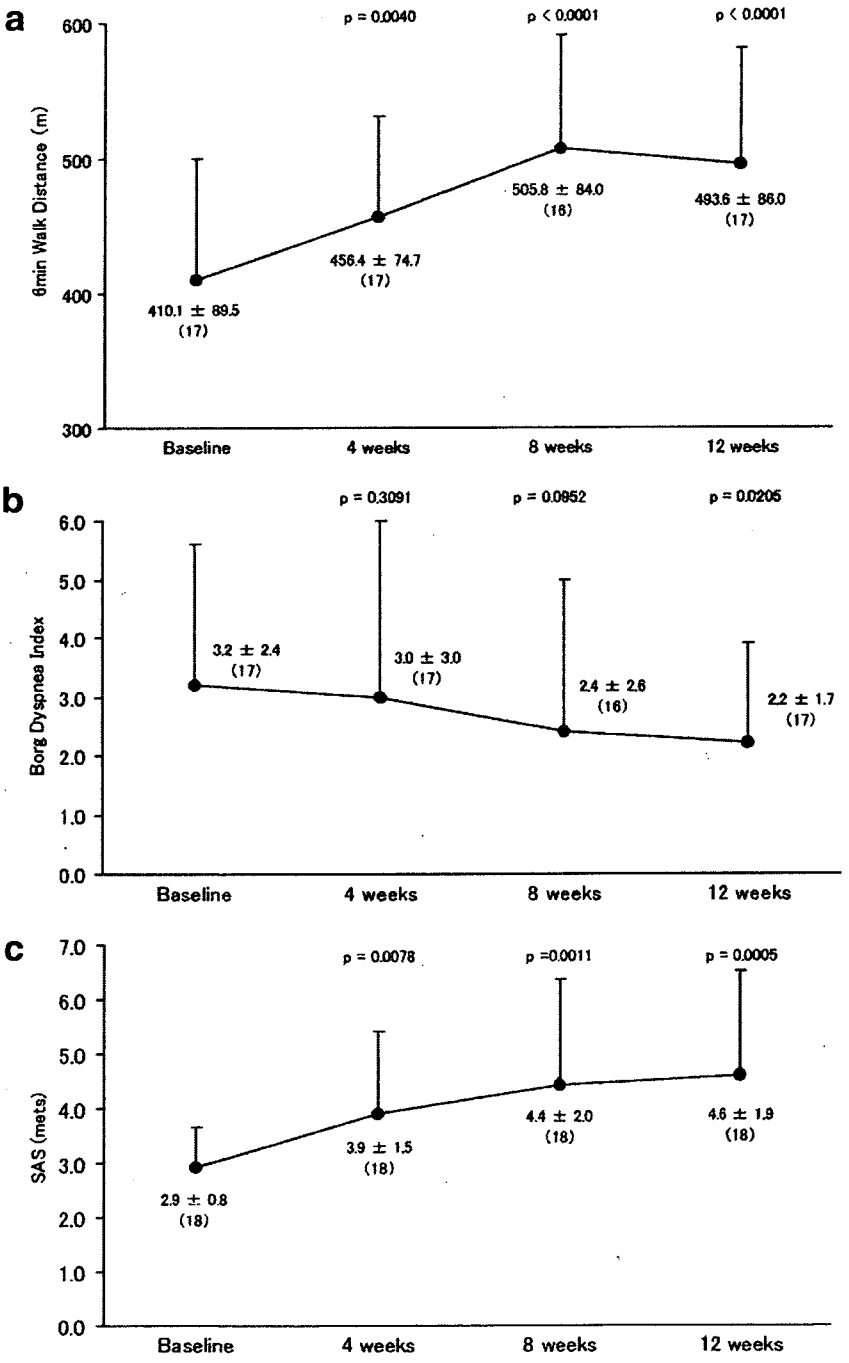


Fig 3. (a) Change in the 6-min walking distance from baseline to week 12. (b) Change in the Borg dyspnea scale from baseline to week 12. (c) Change in the specific activity scale (SAS) from baseline to week 12. Data are expressed as mean± SEM and numbers in parentheses indicate the number of patients assessed.

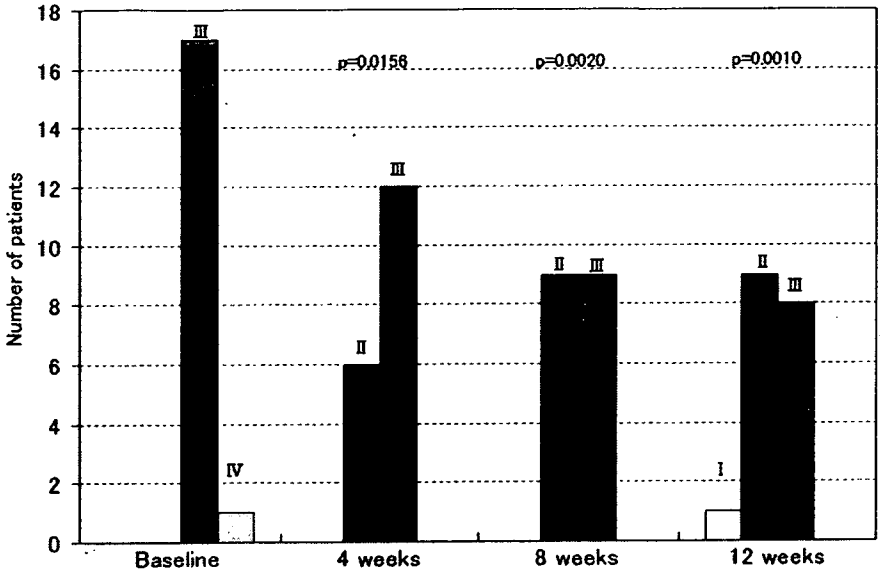


Fig 4. Change in World Health Organization (WHO) functional class from baseline to week 12.

groups. Following administration of bosentan, there were slight and transient decreases in systolic and diastolic blood pressure (5–7 mmHg) but these changes did not appear to be clinically significant. At the end-of-study examination, 4 of 13 Japanese subjects and 2 of 13 Caucasian subjects had alanine aminotransferase (ALAT) values above the upper limit of normal, defined as 23 and 19 U/L in male and female subjects, respectively. The absolute values of ALAT did not exceed 67 U/L in any subject and the increases were not considered clinically significant.

Clinical Study of the Effects of Bosentan in Japanese Patients With PAH

Twenty-one patients were recruited from 11 centers. One patient was excluded from the analysis of efficacy because hemodynamic data at week 12 were not available and another 2 patients were excluded because of technical problems that precluded an accurate measurement of hemodynamic parameters. Therefore, 18 patients (2 males, 16 females), 13 with PPH and 5 with PAH (4 secondary to SLE and 1 secondary to mixed connective tissue disease) were finally included in the analysis of efficacy and 21 were assessed for safety. The mean age was 36±10 years (range, 21–60 years).

After 12 weeks of treatment with bosentan, PVR decreased from 1,281±717 to 899±520 dyn·s/cm^5 ($p<0.0004$). Improvements in other hemodynamic parameters were also observed; for example, mPAP reduced from 52.4±13.8 to 46.8±13.8 mmHg ($p<0.0034$), and CI increased from 2.20±0.74 to 2.61±0.72 L·min^{-1}·m^{-2} ($p<0.0021$) (Fig 2, Table 2). Systolic blood pressure was reduced from 113.0±13.3 to 106.6±9.7 mmHg and diastolic blood pressure from 72.7±11.6 to 66.2±5.2 mmHg, but neither of these changes reached statistical significance. No cases of clinically significant hypotension were observed during the study.

After 12 weeks of treatment, the distance walked in 6 min increased by 83.5±64.1 m ($p<0.0001$) (Fig 3a) and the changes in the Borg dyspnea index paralleled the improvements observed in the 6-min walk test; that is, the index decreased gradually from 3.2±2.4 to 2.2±1.7 throughout the study period, but the changes reached statistical significance only at week 12 ($p=0.0205$) (Fig 3b). The SAS values averaged 2.9±0.8 METs at baseline and increased continuously and significantly, reaching 4.6±1.9 METs at the final assessment ($p=0.0005$) (Fig 3–3). At the beginning of the study, 17 patients were in WHO Class III and 1 in Class IV, but by the end of the study 10 patients had improved to Class I or II ($p=0.0010$) (Fig 4), leaving 8 patients in Class III.

Bosentan, at a dose of 125 mg twice daily, was well tolerated. Adverse drug reactions (excluding unrelated) were observed in 14 of 21 patients (66.7%), including headache (38.1%), dizziness (19.0%), and myalgia (14.3%). Abnormal values of laboratory tests were noted in 10 patients. Bosentan treatment was associated with an increase in aspartate aminotransferase and ALAT (38.1%), an increase in bilirubin (14.3%), a decrease in hemoglobin (14.3%) and a decrease in leukocytes (14.3%). Of 8 patients who had increases in liver aminotransferase concentrations, 3 had concentrations more than 3-fold the upper limit of normal, necessitating discontinuation of the study medication in 1 case. In the other 2 cases, the aminotransferase concentrations returned to normal without discontinuation of treatment, continuing either at the same dose or at a reduced dose of 62.5 mg twice daily. In the other 5 cases, aminotransferase concentrations did not increase more than twice the upper limit of the normal range and returned to the normal range by the end of the study in 4 cases without dose adaptation.

Discussion

Pulmonary arterial hypertension is rapidly progressive, leading to right heart failure and death in a median of 2.8 years from diagnosis. For the majority of cases, the treatments so far developed have been only palliative and the limited oral treatment options include long-term anticoagulant therapy and therapy with calcium-channel blockers, prostacyclin analogues, or phosphodiesterase inhibitors.[19,20] The introduction of intravenous epoprostenol in 1990s greatly improved survival, but this treatment is expensive, the dosage required to sustain these effects increases with time, adverse effects are frequent because of pump malfunction, catheter-related infections and thrombosis, or the

drug induces significant side effects. The efficacy of epoprostenol analogues that can be inhaled (iloprost) or administered orally (beraprost) remains to be confirmed.[21]

It has been recently suggested that local production of ET-1 plays a pathogenic role in PAH, as evidenced by its high plasma concentrations in patients with PPH or PAH,[7,22] the increased expression of ET-1 in the lungs of patients with pulmonary hypertension[6] or idiopathic pulmonary fibrosis.[23] Endothelin-1 has 2 receptors, A and B. Activation of ETA receptors produces vasoconstriction and smooth muscle growth, whereas activation of ETB receptors induces nitric oxide production and vasodilation. Therefore, development of an ET-receptor blocker specific for ETA appears to be desirable. On the other hand, because the ETB receptor mediates release of aldosterone from the adrenal cortex,[24] nonselective blockade of both ETA and ETB receptors may have additional benefits by inhibiting collagen synthesis. Bosentan is an orally effective, nonselective antagonist of ETA and ETB receptors and recent clinical trials have documented promising results in patients with severe pulmonary hypertension,[9,10,18] although its effects are yet to be well characterized in Japanese subjects.

Numerous clinical studies have shown that ethnic groups may differ in their responsiveness to drugs,[25–27] and it has also been suggested that racial differences in the catalytic activity of cytochrome P450 (CYP) isozyme may be responsible for the differences in drug kinetics.[28] The International Conference on Harmonization guideline (ICHE5) document "Ethnic Factors in the Acceptability of Foreign Data" recommends the measurement of pharmacokinetic/pharmacodynamic parameters to permit the clinical effects obtained in one population to be extrapolated to a different population.[13] Ethnic differences in the drug pharmacokinetics depend on gut metabolism/transport and most commonly on hepatic first pass metabolism, but the ethnic differences in hepatic metabolism are known to be unpredictable by race and specific enzyme.[13] The present study showed that the pharmacokinetics of bosentan at the dose of 125 mg are similar in Caucasian and Japanese subjects. Bosentan is metabolized by CYP2C9 and CYP3A4 to 3 metabolites and excreted in bile.[29] A study that used healthy volunteers from broadly defined ethnic groups to assess the adenine to guanine transition in the 5' promoter region of the CYP3A4 gene in a sequence motif known as the nifedipine-specific element, indicated considerable racial differences in the frequency of this polymorphism between Caucasian and Japanese subjects, but there was no ethnic difference in the rate of CYP3A4-dependent drug metabolism and this promoter region polymorphism was considered not to play a major role in determining constitutive CYP3A4 expression.[30] When differences in CYP3A activity between Caucasian and Japanese subjects were assessed using midazolam as an in vivo probe, no statistically significant or clinically important interracial/ethnic difference was observed.[31] Therefore, we assumed that no dose adjustment was necessary when bosentan was used to treat Japanese patients and conducted the first open-label clinical trial of bosentan at the same dose as used in the previous studies carried out in Western populations.

This study demonstrated that 12 weeks of treatment with bosentan at a dose of 125 mg twice daily resulted in significant improvement in symptoms as measured by Borg dyspnea index, exercise capacity as assessed by the 6-min walk test and the SAS, together with an improvement in hemodynamic parameters. The changes in the 6-min walking distance and Borg dyspnea index indicated that patients were able to walk further with less dyspnea; however, the standard deviation of both parameters was greater than the absolute differences from the baseline values to those at the conclusion of the study at 12 weeks, leading to difficulty in interpreting the efficacy of the treatment.[32]

Because patients with cardiopulmonary disorders are usually more symptomatic during exertion, the most direct approach to an evaluation of functional capacity is to inquire about symptoms at rest and during exertion. The majority of exercise tests are designed to evaluate exercise performance at maximal workloads, but daily activities do not generally require energy expenditure in the maximal range. The SAS that we used in the present study quantitatively expresses exercise capacity in terms of energy cost of physical activities and this scale has been shown to linearly correlate with peak oxygen consumption. The reproducibility of measurement was substantial with a mean difference of 0.4±0.5 METs in interobserver variability,[17] prompting us to consider changes greater than 1 MET as clinically relevant. In the present study, SAS increased continuously and significantly throughout the study period, the mean change of 1.7±1.4 indicating a significant treatment effect in favor of bosentan.

In the placebo-controlled studies reported in the literature, treatment with 125 mg of bosentan twice daily was not associated with significant adverse events when compared with placebo.[9,10] However, increased doses led to a frequent elevation of aminotransferase concentrations in accord with the known incidence of abnormal hepatic function.[10] In the present study, 3 patients had increases in aminotransferase with bosentan at a dose of 125 mg twice daily and another 4 patients and 1 patients had increases at doses of 62.5 mg twice daily and 62.5 mg once daily, respectively. In those cases, the abnormal hepatic function progressively improved during bosentan therapy continued at either the same dose or at a reduced dose, except for one case in whom drug withdrawal was necessary. Liver injury induced by bosentan and its metabolites is thought to be mediated through inhibition of the canalicular bile salt export pump (BSEP), as evidenced by a dose-dependent increase in serum bile salts and alkaline phosphatase concentrations in a significant percentage of bosentan-treated patients, the increased cholestatic potency of bosentan with concomitant administration of a known BSEP inhibitor, the reproduction of similar effects in the experimental setting, or in vitro observation of inhibition of BSEP-mediated taurocholate transport by bosentan and metabolites.[33] Recently, it has also been reported that individual differences in susceptibility to the development of intrahepatic cholestasis observed during pregnancy are related to genetic variability in the gene encoding the BSEP.[34] Therefore, if detection of the responsible BSEP and other transporter polymorphisms becomes possible in future, individual susceptibility to drug-induced hepatotoxicity may be predicted.

In conclusion, there are no ethnic differences in the pharmacokinetics of bosentan, and dose adjustment is not necessary for Japanese patients. Japanese patients with severe pulmonary hypertension showed a significant improvement in cardiopulmonary hemodynamics, symptoms, and functional capacity over a 12-week treatment regimen of bosentan 125 mg twice daily. Aminotransferase concentrations were elevated in some cases but mostly returned to normal without discontinuation of therapy. Therefore, bosentan 125 mg twice daily is considered the clinically

preferable dose and is a valuable treatment option for Japanese patients with pulmonary hypertension, though close monitoring of liver function is necessary.

Acknowledgments

We gratefully acknowledge the assistance of Motonori Hatta in the statistical analysis and Dr Andreas Port for his valuable support in the pharmacokinetic study.

References

1. Rubin LJ. Primary pulmonary hypertension. *N Engl J Med* 1997; **336:** 111–117.
2. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al. Primary pulmonary hypertension: A national prospective registry. *Ann Intern Med* 1987; **107:** 216–223.
3. Humbert M, Nunes H, Sitbon O, Parent F, Herve P, Simonneau G. Risk factors for pulmonary arterial hypertension. *Clin Chest Med* 2001; **22:** 459–475.
4. Barst RJ, Rubin LJ, McGoon MD, Caldwell EJ, Long WA, Levy PS. Survival in primary pulmonary hypertension with long-term continuous prostacyclin. *Ann Intern Med* 1994; **1212:** 409–415.
5. Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, et al. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. *N Engl J Med* 1996; **334:** 296–301.
6. Giaid A, Yanagisawa M, Langeleben D, Michel RP, Levy R, Shennib H, et al. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med* 1993; **328:** 1732–1739.
7. Stewart DJ, Levy RD, Cernacek P, Langleben D. Increased plasma endothelin-1 in pulmonary hypertension: Marker or mediator of disease? *Ann Intern Med* 1991; **114:** 464–469.
8. Williamson DJ, Wallman LL, Jones R, Keogh AM, Scroope F, Penny R, et al. Hemodynamic effects of bosentan, an endothelin receptor antagonist, in patients with pulmonary hypertension. *Circulation* 2000; **102:** 411–418.
9. Channick RN, Simonneau G, Sitbon O, Robbins IM, Frost A, Tapson VF, et al. Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: A randomized placebo-controlled study. *Lancet* 2001; **358:** 1119–1123.
10. Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, et al. Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med* 2002; **346:** 896–903.
11. Weber C, Schmitt R, Birnboeck H, Hopfgartner G, Eggers H, Meyer J, et al. Multiple-dose pharmacokinetics, safety, and tolerability of bosentan, an endothelin receptor antagonist, in healthy male volunteers. *J Clin Pharmacol* 1999; **39:** 703–714.
12. Dingemanse J, van Giersbergen PLM. Clinical pharmacology of bosentan, a dual endothelin receptor antagonist. *Clin Pharmacokinet* 2004; **43:** 1089–1115.
13. Johnson JA. Predictability of the effects of race or ethnicity on pharmcokinetics of drugs. *Int J Clin Pharmacol Ther* 2000; **38:** 53–60.
14. Dell D, Lausecker B, Hopfgartner G. Evolving bioanalytical methods for the cardiovascular drug bosentan. *Chromatographia* 2002; **55**(Suppl): S115–S119.
15. Gibaldi M, Perrier D. Pharmacokinetics, 2nd edn. New York: Marcel Dekker; 1982.
16. Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982; **14:** 377–381.
17. Sasayama S, Asanoi H, Ishizaka S, Miyagi K. Evaluation of functional capacity of patients with congestive heart failure. *In*: Yasuda H, Kawaguchi H, editors. New aspects in the treatment of failing heart. Tokyo: Springer-Verlag; 1992; 113–117.
18. Channick RN, Rubin LJ. Endothelin receptor antagonism: A new era in the treatment of pulmonary arterial hypertension. *Pulm Hypertens* 2002; **1:** 13–17.
19. Runo RR, Loyd JE. Primary pulmonary hypertension. *Lancet* 2003; **361:** 1533–1544.
20. Hashimoto K, Nakamura K, Fujio H, Miyaji K, Morita H, Kusano K, et al. Epoprosterol therapy decreases elevated circulating levels of monocyte chemoattractant protein-1 in patients with primary pulmonary hypertension. *Circ J* 2004; **68:** 227–231.
21. Ono F, Nagaya N, Kyotani S, Oya H, Nakanishi N, Miyatake K. Hymodynamic and hormonal effects of beraprost sodium, an orally active prostacyclin analogue, in patients with secondary precapillary pulmonary hypertension. *Circ J* 2003; **67:** 375–378.
22. MacLean MR. Endothelin-1: A mediator of pulmonary hypertension? *Pulm Pharmacol Ther* 1998; **11:** 125–132.
23. Saleh D, Furukawa K, Tsao MS, Maghazachi A, Corrin B, Yanagisawa M, et al. Elevated expression of endothelin-1 and endothelin-converting enzyme-1 in idiopathic pulmonary fibrosis: Possible involvement of proinflammatory cytokines. *Am J Resp Cell Mol Biol* 1997; **16:** 187–193.
24. Pecci A, Cozza EN, Devlin M, Gomez-Sanchez CE, Gomez-Sanchez EP. Endothelin-1 stimulation of aldosterone and zona glomerulosa ouabain-sensitive sodium/potassium-ATPase. *J Steroid Biochem Mol Biol* 1994; **50:** 49–53.
25. Zhou HH, Koshakji RP, Silberstein DJ, Wilkinson GR, Wood AJ. Altered sensitivity to and clearance of propranolol in men of Chinese descent as compared with American whites. *N Engl J Med* 1989; **320:** 565–570.
26. Yamasaki Y, Sakamoto K, Watade H, Kajimoto Y, Hori M. The Arg 192 isoform of paraoxonase with low sarin-hydrolyzing activity is dominant in the Japanese. *Hum Genet* 1997; **101:** 67–68.
27. Kim RB, Yamazaki H, Chiba K, O'Shea D, Mimura M, Guengerich FP, et al. In vivo and in vitro characterization of CYP2E1 activity in Japanese and Caucasians. *J Pharmacol Exp Ther* 1996; **279:** 4–11.
28. Johnson JA, Herring VL, Wolfe MS, Relling MV. CYP1A2 and CYP2D6 4-hydroxylate propranolol and both reactions exhibit racial differences. *J Pharmacol Exp Ther* 2000; **294:** 1099–1105.
29. Weber C, Gasser R, Hopfgartner G. Absorption, excretion, and metabolism of the endothelin receptor antagonist bosentan in healthy male subjects. *Drug Metab Disp* 1999; **27:** 810–815.
30. Ball SE, Scatina J, Kao J, Ferron GM, Fruncillo R, Mayer P, et al. Population distribution and effects on drug metabolism of a genetic variant in the 5' promoter region of CYP3A4. *Clin Pharmacol Ther* 1999; **66:** 288–294.
31. Tateishi T, Watanabe M, Nakura H, Asoh M, Shirai H, Mizorogi Y, et al. CYP3A activity in European American and Japanese men using midazolam as an in vivo probe. *Clin Pharmacol Ther* 2001; **65:** 333–339.
32. Pareira BN, Salvi S. Bosentan for pulmonary hypertension. *N Engl J Med* 2002; **347:** 292.
33. Fattinger K, Funk C, Pantse M, Weber C, Reichen J, Stieger B, et al. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: A potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther* 2001; **69:** 223–231.
34. Eloranta ML, Hakli T, Hiltunen M, Helisalmi S, Punnonen K, Heinonen S. Association of single nucleotide polymorphisms of the bile salt export pump gene with intrahepatic cholestasis of pregnancy. *Scand J Gastroenterol* 2003; **38:** 648–652.

Appendix 1

Investigators in the Study

Chiba University: Yasunori Kasahara, Nobuhiro Tanabe; Juntendo University: Hiroshi Hashimoto, Yoshiaki Tokano, Ken Yamaji, Katsumi Miyauchi; Keio University: Satoshi Ogawa, Toru Satoh; Kitasato University: Hirobumi Kondo, Takamoto Inomata, Hirobumi Kondo, Jun Okada; Fujita Health University: Shunji Yoshida; National Cardiovascular Center: Norifumi Nakanishi, Shingo Kyotani; Okayama University: Tohru Ohe, Hiromi Matsubara; Yamaguchi University: Kazuya Murata, Takahumi Hiro; Kurume University: Tsutomu Imaizumi, Nobuhiro Tahara; Nagasaki University Shigeru Kohno, Yoshiyuki Miyahara, Soji Ikeda; Kagoshima University, Chuwa Tei, Shinichi Minagoe.

Prevention of Left Ventricular Remodeling by Long-Term Corticosteroid Therapy in Patients With Cardiac Sarcoidosis

Chiung-Zuan Chiu, MD, Satoshi Nakatani, MD, Guican Zhang, MD, Teruo Tachibana, MD, Fumio Ohmori, MD, Masakazu Yamagishi, MD, Masafumi Kitakaze, MD, Hitonobu Tomoike, MD, and Kunio Miyatake, MD

Forty-three patients with cardiac sarcoidosis were studied echocardiographically before and after (mean follow-up 88 months) steroid therapy to determine the effectiveness of corticosteroids to prevent left ventricular (LV) remodeling and improve LV contractility. In patients with initial LV ejection fractions (LVEFs) ≥55%, long-term steroid therapy showed preventive effects for LV remodeling and LV function. Patients with LVEF <54% showed significant reductions of LV volumes and LVEF improvement. However, in patients with LVEFs <30%, steroid therapy resulted in neither LV volume reductions nor improved LVEFs. In the early or middle stage of the disease, steroid therapy may be protective or therapeutic in preventing LV remodeling and preserving LV function. However, it may not be as effective in the late stage. ©2005 by Excerpta Medica Inc.

(Am J Cardiol 2005;95:143–146)

Sarcoidosis is a systemic granulomatous disease of undefined cause involving multiple organs.[1] Although its cardiac involvement has been demonstrated in 20% to 50% in autopsy studies, clinical cardiac manifestations have been seen in only about 5% of patients with sarcoidosis.[1–3] Cardiac sarcoidosis has significant morbidity and mortality due to fatal arrhythmia, atrioventricular conduction disturbance, and refractory congestive heart failure, but its diagnosis has not always been easy.[4,5] Therefore, early suspicion and diagnosis of cardiac sarcoidosis should be desirable in patients with cardiac symptoms[6,7] with abnormal echocardiographic or scintigraphic findings.[8–10] In patients with definite diagnoses or strong probabilities of cardiac sarcoidosis, corticosteroid therapy should be started, even if myocardial biopsy results are negative.[1,3] Previous reports have shown that steroid therapy for sarcoidosis is more effective for the heart than for other organs.[11,12] Although the long-term benefit of steroid therapy in reducing clinical morbidity and mortality has been demonstrated recently,[13] the effect of steroids on left ventricular (LV) morphology and function has not been defined clearly. Using echocardiography, we investigated if steroid therapy could prevent LV remodeling and improve LV function.

• • •

Patients who were diagnosed with cardiac sarcoidosis and underwent long-term steroid therapy in our institution from January 1990 to June 2001 were analyzed retrospectively. We diagnosed patients with cardiac sarcoidosis according to the proposal by the Specific Diffuse Pulmonary Disease Research Group, Sarcoidosis Division (Japanese Ministry of Health and Welfare).[8,9,14,15] Briefly, the diagnostic criteria consist of histologic confirmation and electrocardiographic, echocardiographic, or myocardial scintigraphic abnormalities compatible with cardiac sarcoidosis. After excluding 9 patients without steroid therapy or regular follow-up, 43 patients met these criteria (16 men and 27 women; mean age 48 ± 14 years, range 21 to 71). All patients had coronary angiography, and those who had coronary heart disease were excluded. Right ventricular endomyocardial biopsies were performed on 28 patients, and 12 showed positive results. Pulmonary involvement was noted in 11 patients, skin lesions in 10, and eye involvement in 14. The occurrence of cardiac events (conduction disturbance, lethal arrhythmia, congestive heart failure, and sudden cardiac death) and mortality were investigated during follow-up.

All patients underwent echocardiographic examinations before and after steroid therapy (88 ± 48 months, range 1 to 196) with commercially available ultrasound systems. LV end-diastolic and end-systolic dimensions were determined from M-mode or B-mode echocardiograms. LV end-diastolic and end-systolic volumes and LV ejection fractions (LVEFs) were measured by the modified Simpson's method. LV volumes were corrected by body surface area to obtain LV volume indexes.

All patients were given prednisolone every other day according to a standard protocol.[13] The starting dose of prednisolone was 60 mg every other day for 2 months, which was tapered gradually to the final maintenance dose of 10 mg every other day.

All data are expressed as mean ± SD. Statistical analyses were performed using SPSS 10.0 software (SPSS, Inc., Chicago, Illinois). Comparisons of

From the Department of Cardiology, National Cardiovascular Center, Osaka, Japan; Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan; Union Hospital, Fuzhou, People's Republic of China; Kanpo Health Office, Osaka, Japan; and Sharp Medicala Office, Osaka, Japan. This work was supported by the Grant for Cardiovascular Diseases from the Japanese Ministry of Health, Labor and Welfare, Tokyo, Japan. Dr. Nakatani's address is: Department of Cardiology, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. E-mail: nakatas@hsp.ncvc.go.jp. Manuscript received June 23, 2004; revised manuscript received and accepted August 26, 2004.

0002-9149/05/$–see front matter
doi:10.1016/j.amjcard.2004.08.083

TABLE 1 Patient Characteristics

Characteristic	All	Group A (LVEF ≥55%, n = 22)	Group B (30 ≤LVEF <55%, n = 10)	Group C (LVEF <30%, n = 11)	p Value
Age (yrs)	48 ± 14	44 ± 15	45 ± 9	58 ± 9	0.018
Men/women	16/27	10/12	3/7	3/8	0.533
Patients with myocardial biopsy	28	10	8	10	
Patients with positive biopsy results		3 (33%)	4 (50%)	5 (50%)	
LVEF (%)	51 ± 22	69 ± 7	40 ± 10	22 ± 7	<0.0001
LV end-diastolic diameter (mm)	53 ± 10	46 ± 4	56 ± 7	64 ± 7	<0.0001
LV end-systolic diameter (mm)	39 ± 14	29 ± 3	45 ± 7	58 ± 7	<0.0001
LVEDVI (ml/m^2)	85 ± 43	59 ± 19	92 ± 19	133 ± 42	<0.0001
LV end-systolic volume index (ml/m^2)	49 ± 33	19 ± 5	57 ± 22	104 ± 37	<0.0001

TABLE 2 Comparison of Echocardiographic Parameters Before and After Steroid Therapy

Parameter	Before Steroid Therapy	After Steroid Therapy	p Value
LVEF (%)	51 ± 22	52 ± 23	0.127
LV end-diastolic diameter (mm)	53 ± 10	54 ± 10	0.445
LV end-systolic diameter (mm)	39 ± 14	39 ± 15	0.978
LVEDVI (ml/m^2)	85 ± 43	82 ± 41	0.371
LV end-systolic volume index (ml/m^2)	49 ± 33	47 ± 32	0.448

continuous variables between 2 groups were made by the unpaired Student's *t* test. Comparisons of variables before and after steroid therapy were made by the paired Student's *t* test. The analysis of variance test was used for comparisons among 3 groups. Long-term survival was estimated by Kaplan-Meier analysis, and differences in survival were assessed using a log-rank test. Bivariate analysis of the correlations was performed by Pearson's correlation coefficient analysis. A p value <0.05 was considered statistically significant.

Basic data and echocardiographic parameters are listed in Table 1. Patients were divided into 3 groups according to the LVEF before steroid therapy: group A (n = 22), normal LVEFs (≥55%); group B (n = 10), mildly to moderately reduced LVEFs (30% to 54%); and group C (n = 11), severely reduced LVEFs (<30%). Patients in group C were older than those in the other 2 groups (p = 0.018). LV diameters and volumes became larger as LV function deteriorated. Bivariate analysis showed that age was significantly positively correlated with LV end-systolic diameter (r = 0.396, p = 0.012) and LV end-systolic volume index (r = 0.339, p = 0.032) and negatively correlated with LVEF before steroid therapy (r = −0.427, p = 0.006), suggesting that cardiac sarcoidosis tended to be more advanced in older patients.

Overall, there were no significant differences in LV volumes and function before and after steroid therapy in all patients (Table 2). However, when we examined the data in each group, they showed different courses of changes (Figure 1). In group A, the LV end-diastolic volume index (LVEDVI) decreased (from 59 ± 19 to 57 ± 19 ml/m^2, p = 0.347) and the LVEF did not change (69 ± 7% to 69 ± 5%, p = 0.277) after long-term steroid therapy. In group B, however, the LVEDVI decreased significantly (from 92 ± 29 to 78 ± 25 ml/m^2, p = 0.018) and the LVEF increased significantly (from 40 ± 10% to 51 ± 12%, p = 0.008) at follow-up. In group C, neither the LVEDVI (133 ± 42 to 134 ± 33 ml/m^2, p = 0.918) nor the LVEF (22 ± 7% to 19 ± 5%, p = 0.082) changed substantially.

Because echocardiographic data before steroid therapy were different among the 3 groups by definition, we calculated percent changes of each parameter. The percent change in the LVEDVI after steroid therapy was most remarkable in group B compared with the other groups, although it did not reach statistical significance (−2 ± 10% in group A, −15 ± 13% in group B, and 7 ± 38% in group C, p = 0.125; Figure 2). The percent change of the LVEF was significantly larger in patients in group B than those in the other groups (3 ± 9% in group A, 28 ± 20% in group B, and −10 ± 21% in group C, p <0.0001; Figure 2), suggesting that patients in group B benefited the most from steroid therapy.

Fourteen patients required permanent pacemakers, and 3 patients required implantable-cardioverter defibrillators. Angiotension-converting enzyme inhibitors were administered to 9 patients in group B and to 6 patients in group C. Beta blockers were given to 4 patients in group B and to 7 patients in group C. Intravenous catecholamines were used in 9 patients in group C who had severe refractory congestive heart failure. The total survival rate for all 43 patients was relatively good, at 98% after 1 year, 93% after 3 years, 90% after 5 years, and 84% after 10 years. However, when analyzed on a subgroup basis, there was a difference (Figure 3). There were no cardiac deaths in group A during the follow-up period of 10 years. The survival rate was 100% in group B after 1, 3, and 5 years and 67% after 10 years. The survival rate in group C was clearly lower: 91% after 1 year, 72% after 3 years, 57% after 5 years, and 19% after 10 years. A log-rank test revealed a significant difference in the long-term survival rate between groups A and C (log-rank 16.470, p <0.0001) and between groups B

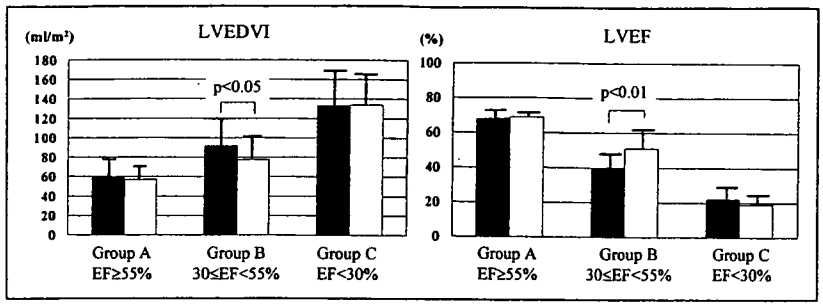


FIGURE 1. Comparison of the LVEDVI and LVEF among 3 groups. *Black and white columns*, before and after steroid therapy, respectively.

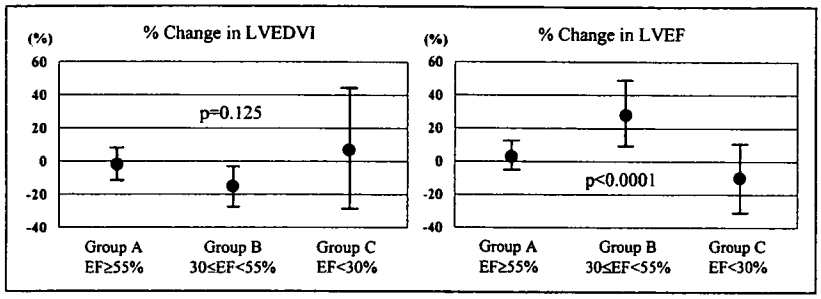


FIGURE 2. Comparison of percent changes in the LVEDVI and LVEF among 3 groups.

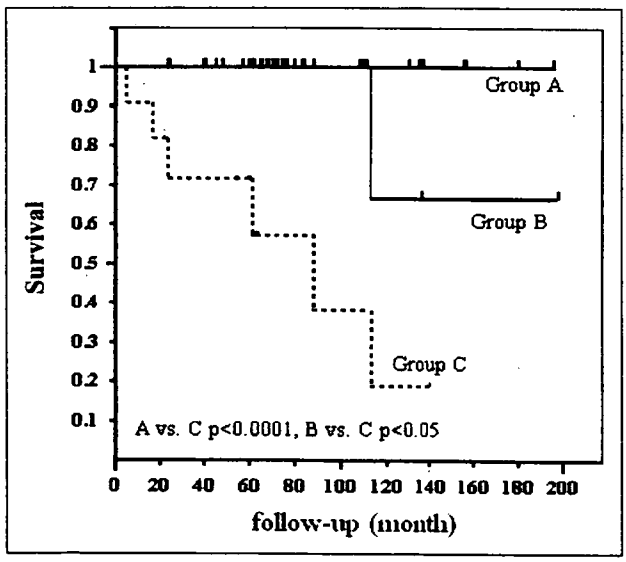


FIGURE 3. Comparison of survival curves among the 3 groups.

and C (log-rank 4.683, $p < 0.05$). After age matching, the log-rank test still revealed a significant difference in survival between groups A and C (log-rank 10.482, $p < 0.005$) and a marginal difference between groups B and C (log-rank 3.811, $p = 0.051$).

• • •

In the present study, for the first time, we showed that the effect of corticosteroid therapy on LV remodeling and function depended on the initial LV size and function and that steroid therapy seemed to have evident response in selected patients. In the early stage of the disease, when LV function and volume were normal, steroid therapy seemed to have the protective effects of preventing LV remodeling and the deterioration of cardiac function. In the middle stage of the disease, the clinical effect of steroid therapy was most obvious and potent, because LV size decreased and LVEF improved significantly. However, in the late stage, steroid therapy appeared not to have a significant beneficial effect, with unchanged LV size and LVEF. The different responses to steroid therapy may reflect the degree of irreversible myocardial damage and fibrosis caused by inflammation.

From the analysis of percent changes in the LVEDVI and LVEF after steroid therapy, it was also demonstrated that patients in the middle stage (group B) had most benefit from steroid therapy. This was supported by the Kaplan-Meier analysis showing better survival in group B than in group C. A recent report has similarly shown that patients with LVEFs >50% had better survival than those with LVEFs <50%.[13]

In the present study, we investigated mortality but not morbidity in patients with sarcoidosis. Patients with cardiac sarcoidosis sometimes present with congestive heart failure or lethal arrhythmia requiring hospitalization. Further, steroid therapy always has risks for infection, bleeding from peptic ulcers, osteoporosis, and so on.[12] Although we showed the prevention of LV remodeling by early steroid therapy, one should always pay attention to these possible complications. We found that patients in the early stage showed no substantial changes in LV size and function after corticosteroid therapy. However, we could not tell if this was due to the effect of steroid therapy or the natural course of the disease. To clarify this, another prospective study should be performed in which patients are randomly given steroids or a placebo. This was a retrospective study, and only patients with echocardiographic studies before and after long-term steroid therapy were enrolled. Therefore, some patients with suboptimal echocardiographic images or those without regular echocardiographic follow-up were excluded, resulting in a relatively small number of patients.

•

Acknowledgment: We thank Wei-Chih Hsu, MD, Section of Neurology, Shin-Kong Wu Ho-Su Memorial Hospital, for his assistance in statistical analysis. Also, we thank Chikao Yutani, MD, Director, Department of Pathology, for his thoughtful suggestions in preparing this report.

1. Newman LE, Rose CS, Maier LA. Sarcoidosis. *N Engl J Med* 1997;336:1224–1234.
2. Loncope WT, Freiman DGH. A study of sarcoidosis: based on a combined investigation of 160 cases including 30 autopsies from the Johns Hopkins Hospital and Massachusetts General Hospital. *Medicine* 1952;31:1–132.