Treatment of Dilated Cardiomyopathy With Electroporation of Hepatocyte Growth Factor Gene Into Skeletal Muscle

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Abstract—Hepatocyte growth factor (HGF) is a potent angiogenic and antifibrotic factor. Cardioprotective effects of HGF for idiopathic dilated cardiomyopathy were examined in hamsters with electroporation of plasmid DNA into skeletal muscle. We used hamster skeletal muscle as a protein producer of HGF gene. A plasmid vector encoding HGF (HGF group, n=12) or empty plasmid (placebo group, n=12) was transferred with in vivo electroporation into tibialis anterior muscles of hamsters with inherited dilated cardiomyopathy (TO-2 strain). The HGF group had greater serum HGF levels (21.6±2.2 versus 0.11±0.07 ng/mL, $P<0.05$), higher left ventricular ejection fraction (47.9±9.4% versus 28.8±11.2%, $P<0.05$), and greater wall thickening (31.6±6.3% versus 19.7±6.1%, $P<0.05$) when compared with the placebo group. The HGF group had smaller areas of ventricular fibrosis (11.8±3.4% versus 17.1±3.5%, $P<0.05$) and lower hydroxyproline content (3.7±0.7 versus 5.1±0.9 μmol/g, $P<0.05$) than did the placebo group. The HGF group also had higher capillary density (1885±232 versus 1447±182 vessel/mm^2, $P<0.05$) and higher matrix metalloprotease-1 activity (13.1±3.5 versus 8.1±3.6 μg/collagen degraded per hour per gram tissue, $P<0.05$) than did the placebo group. Exogenous HGF might improve the deleterious changes in myocardial function and structure in the hamster with dilated cardiomyopathy. Systemic delivery of gene products with in vivo electroporation into skeletal muscle seemed to be an alternative means of direct gene delivery. (***Hypertension*. 2004;44:365-371.**)

Key Words: cardiomyopathy ■ genes ■ growth substances ■ hamsters ■ heart failure

Dilated cardiomyopathy is one of the major causes of severe heart failure and is an indication for heart transplantation. Hamsters with inherited dilated cardiomyopathy are a well-known model of human dilated cardiomyopathy.[1,2] Hepatocyte growth factor (HGF) is a mesenchyme-derived pleiotropic factor that has potent angiogenic and antifibrotic action.[3,4] Further, administration of human recombinant HGF prevented fibrosis in liver and pulmonary injury models.[5–7] HGF also has important roles in tumor growth and tumor angiogenesis.[8,9]

Electroporation has been widely used to introduce DNA into various cell types in vitro. Gene transfer by in vivo electroporation has been effective for introducing DNA into animal tissues.[10,11] Electroporation into skeletal muscle has been used for muscular disease[12] and for the systemic delivery of bioactive proteins.[13–16] Gene transfection into skeletal muscle has been used for systemic delivery of therapeutic proteins for liver[6] and cardiac diseases.[17–21] The goal of this study was to test the hypothesis that exogenous HGF protein might improve the deleterious changes in myocardial function and structure in the hamster with dilated cardiomyopathy.

Methods

Animals, Plasmid DNA, and Experimental Protocols

Male cardiomyopathic TO-2 hamsters and healthy FIb hamsters aged 10 weeks were obtained from BIO breeder, Inc (Watertown, Mass). Hamsters were handled according to animal experiment guidelines at our institute. Rat-HGF cDNA cloned by polymerase chain reaction was inserted into the unique *Xho* I site between the cytomegalovirus immediate early enhancer-chicken β-actin hybrid promoter and rabbit β-globin poly A site of the pCAGGS expression plasmid.[3] The resulting plasmid, pCAGGS-*HGF*, was grown in *Escherichia coli* DH5α. The plasmid was purified with plasmid DNA kit (Quiagen). For electroporation of the DNA, we inserted needles into the bilateral anterior tibialis muscles and delivered electrical pulses 6× each at 100 V and 50 ms with an electrical pulse generator (Electro Square Porator T820, BTX).

In a preliminary experiment of electroporation using 10 FIb hamsters, 6 of 10 hamsters had slight plasmid buffer leakage. In the inclusion experiment, we injected plasmid into 25 TO-2 hamsters aged 11 weeks. Four of 25 had buffer leakage. We measured plasma HGF level 3 days after the first electroporation of 800 μg of HGF plasmid (day 0) to determine the success of the procedure. Inclusion criterion was a plasma HGF level >5.0 ng/mL. Twelve TO-2 hamsters treated with HGF fulfilled the criterion and received the following electroporations and examinations.

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Effect of Electroporation (Days 0, 7, 14) on Serum HGF Levels

		Serum HGF, ng/mL		
Group	No.	Day −7	Day 7	Day 21
F1b	12	0.08±0.05	0.09±0.05	0.09±0.06
Placebo	12	0.11±0.07	0.11±0.05	0.13±0.06
HGF	12	0.11±0.08	19.0±2.1*†‡	20.2±2.2*†‡

*Significantly ($P<0.05$) different from respective value in the control F1b group.

†Significantly ($P<0.05$) different from respective value in the placebo group.

‡Significantly ($P<0.05$) different from the value at day −7 in the HGF group.

We administered HGF plasmid (800 μg per animal) to TO-2 hamsters on days 0, 7, and 14 (HGF group, n=12). We administered empty pCAGGS to control TO-2 at similar time points (placebo group, n=12). We used F1b hamsters without any treatment as age-matched normal control (control group, n=12). We determined the changes in serum HGF levels on days −7, 7, and 21 using an ELISA kit and conducted echocardiography for cardiac function (days −7 and 21) and pathology for cellular changes (days 21).

Echocardiography

Echocardiographic studies in each group under anesthesia were performed with leading-edge method on days −7 and 21.[22] Left ventricular (LV) ejection fraction was calculated using the Pombo formula: $(EDD^3-ESD^3)/EDD^3$, where EDD is end-diastolic dimension and ESD is end-systolic dimension. Cardiac output (CO) was calculated as CO=aortic velocity time integral×(π [LV outflow tract/2]^2).[23] Arterial pressure was measured with a polyethylene catheter inserted into the carotid artery after echo-Doppler studies on day 21. Meridional wall stress σ was estimated as σ=arterial pressure×[ID/(1+PWT/ID)], where ID is internal dimension, and PWT is posterior wall thickness.[24] Systemic vascular resistance (SVR) was calculated as SVR=mean arterial pressure/CO.

Pathology and Tissue Biochemistry

Body weight and LV weight were measured on day 21. Transverse sections of the ventricle were stained with hematoxylin-eosin and Masson trichrome staining. Muscle fiber diameter was evaluated in cross sections that included a nuclear profile.[24] A digital image analyzer (Mac SCOPE, Mitani Co.) was used to calculate percent fibrosis area. Capillary density was determined with anti–von Willebrand antibody staining.[25] The hydroxyproline content of the myocardium was measured according to the method described by Green and Reagan.[26] Matrix metalloprotease-1 (MMP-1) was evaluated by a collagenase type I activity test kit.

Statistics

Data are presented as mean±SD. Statistical analysis between the groups was performed by 1-way ANOVA followed by Bonferroni/Dunn method. Differences were considered significant at $P<0.05$.

Results

Serum Levels of HGF After Electroporation

Serum HGF levels on days 7 and 21 were significantly ($P<0.05$) higher in the HGF group than in the placebo and control groups (Table). Serum HGF levels did not differ when comparing the placebo and control groups.

Effect of HGF on Hemodynamics and Myocardial Parameters

There were no significant differences in EDD, LV ejection fraction, and PW thickening between the control, placebo, and HGF groups on day −7 (Figure 1). EDD tended to increase in the placebo group, and EDD was larger in the placebo group than in the HGF and control groups on day 21 (Figures 1 and 2). LV ejection fraction and PW thickening tended to decrease in the placebo and HGF groups, but were higher in the HGF group than in the placebo group on day 21. Thus, treatment by HGF seemed to prevent the development of systolic dysfunction in cardiomyopathy.

Doppler echocardiography revealed higher peak and steeper deceleration of the peak early diastolic filling velocity (E wave), that is, the restrictive pattern of mitral inflow in placebo hamster (Figure 3). In HGF hamsters, amplitudes of E and the peak filling velocity at atrial contraction (A wave) became similar, and E wave steepness became smaller (ie, pseudonormalization pattern of mitral inflow was observed).

E/A ratio was greater in the placebo group than in the control and HGF groups (Figure 3). Isovolumic relaxation time was shorter in the placebo group than in the control and HGF groups. Deceleration time of E wave was shorter in the placebo group than in the control and HGF groups, and deceleration rate of E wave or E wave amplitude divided by deceleration time of E wave was greater in the placebo group than in the control and HGF groups. Thus, treatment by HGF seemed to reverse LV diastolic dysfunction in cardiomyopathy.

Mean arterial pressure ([in mm Hg] F1b 93.9±5.6, placebo 88.9±5.1, HGF 88.4±6.1) and heart rate ([in bpm] F1b 395±26, placebo 403±28, HGF 389±24) were similar among the 3 groups. LV wall stress was significantly higher in the placebo group than in the control and HGF groups (Figure 4). CO was lower in the placebo group than in the control and HGF groups. SVR was higher in the placebo group than in the control and HGF groups. Thus, hemodynamic parameters seemed to be preserved in the HGF group.

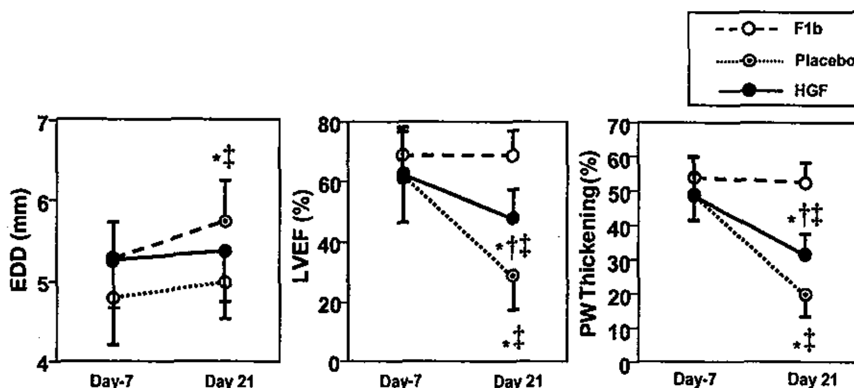

Figure 1. Temporal changes in LV dimension and systolic function measured by echocardiography in each study group. Data are expressed as mean±SD. LVEF indicates left ventricular ejection fraction. *$P<0.05$ vs F1b. †$P<0.05$ vs placebo. ‡$P<0.05$ vs day −7.

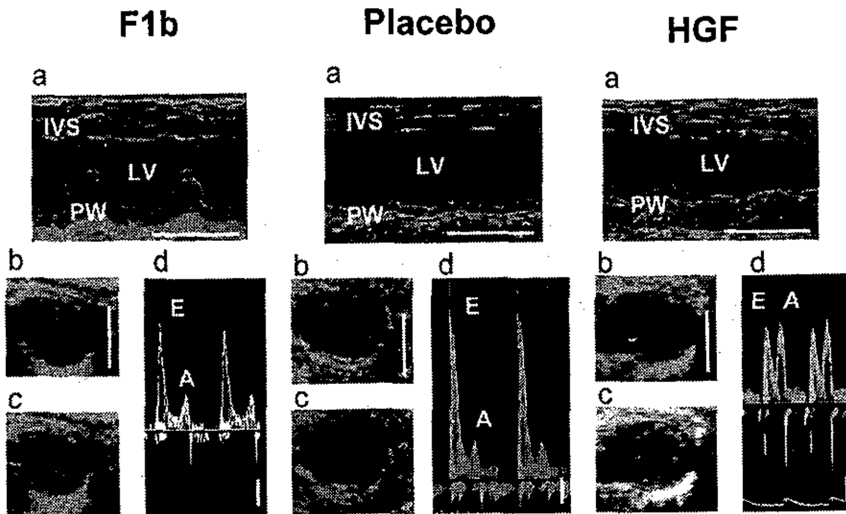

Figure 2. Representative echocardiographic images on day 21 in each study group. a, M-mode views, white bar indicates 200 ms; (b) end-diastolic 2-dimensional short axis views, white bar indicates 5 mm; (c) end-systolic 2-dimensional short axis views; (d) transmitral inflow patterns, white bar indicates 10 cm/s. IVS indicates interventricular septum; LV, left ventricular cavity; E, peak early diastolic filling velocity; A, peak filling velocity at atrial contraction.

There were no differences in body weight among the 3 groups (Figure 5). LV weight/body weight ratio and LV weight/tibial length ratio were significantly higher in the placebo group than in the control group. Thus, the development of LV hypertrophy seemed to be prevented in the HGF group. However, myocardial diameter was similar among the 3 groups. Thus, this LV hypertrophy did not seem to be derived from myofibrillar hypertrophy.

Histological Analysis

Macroscopic imaging revealed LV cavity dilatation and fibrosis in a heart slice from placebo hamster compared with control F1b hamster (Figure 6). A heart slice image from HGF hamster showed smaller LV cavity and less fibrotic area compared with a placebo heart (LV internal diameter [in mm]: control F1b 4.4, placebo 6.3 mm, HGF 4.8 mm). Although fibrosis area was larger in the placebo and HGF groups than in the control group, the increase was attenuated in the HGF group (Figure 7). Tissue hydroxyproline content, an index of fibrosis, was higher in the placebo and HGF groups than in the control, but again, the increase was attenuated in the HGF group. Although myocardial capillary density was lower in the placebo and HGF groups than in the control, the density was higher in the HGF group than in the placebo group. MMP-1 activity was higher in the HGF group than in the control and placebo groups. There was a negative correlation between MMP-1 activity and % fibrosis area ($r=-0.62$, $r^2=0.39$, $P<0.05$). There was a positive correlation between MMP-1 activity and capillary density ($r=0.48$, $r^2=0.24$, $P<0.05$).

Discussion

The present study demonstrated that (1) serum rat-HGF levels increased following in vivo electroporation of rat-HGF plasmid into the skeletal muscle of cardiomyopathic hamsters; (2) LV systolic and diastolic functional deterioration was atten-

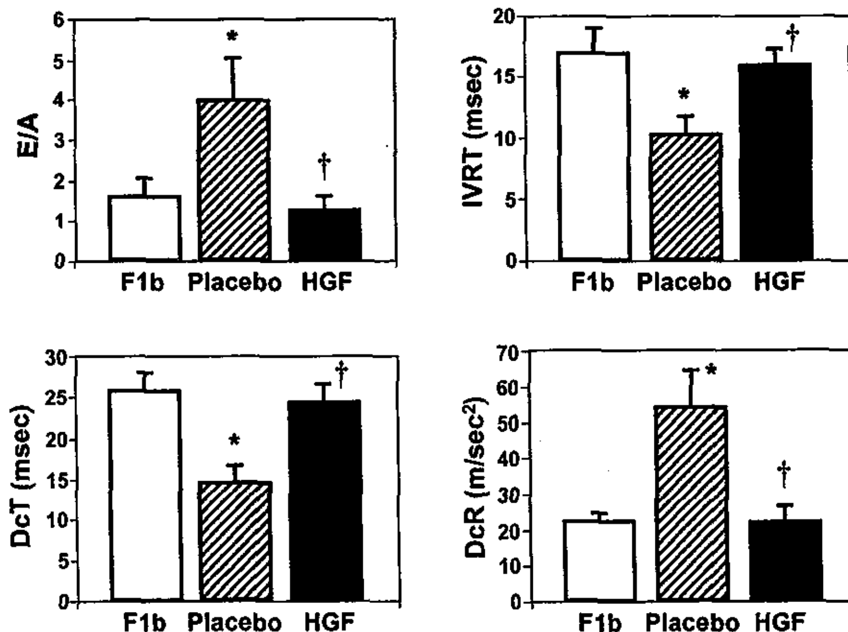

Figure 3. LV diastolic functional parameters determined by Doppler echocardiography on day 21 in each study group. Data are expressed as mean±SD. E indicates peak early diastolic filling velocity; A, peak filling velocity at atrial contraction; IVRT, isovolumic relaxation time; DcT, deceleration time in early diastolic filling velocity; DcR, deceleration rate in early diastolic filling velocity. *$P<0.05$ vs F1b. †$P<0.05$ vs placebo.

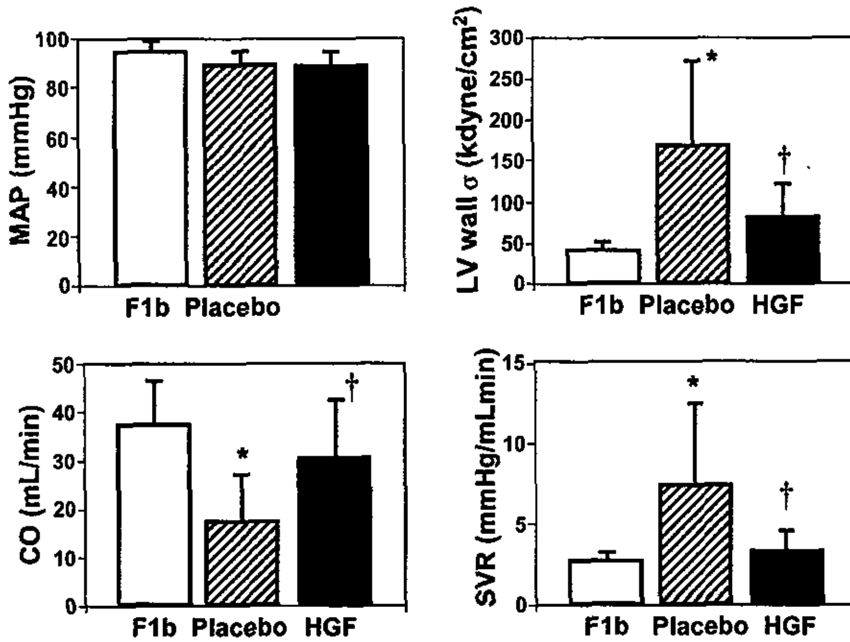

Figure 4. Hemodynamic parameters on day 21 in each study group. Data are expressed as mean±SD. MAP indicates mean arterial pressure; σ, stress. *$P<0.05$ vs F1b. †$P<0.05$ vs placebo.

uated in cardiomyopathic hamsters treated with HGF; (3) the extent of cardiac hypertrophy and fibrosis was attenuated in cardiomyopathic hamsters treated with HGF; and (4) myocardial capillary density and MMP-1 activity were higher in the myocardium of the hamsters treated with HGF than in that of hamsters given placebo. HGF has been known to possess a remarkable potential for angiogenesis. Systemic HGF might have decreasing effects on afterload via the neoangiogenesis and vascular dilatation. However, in the present study, improvements in cardiac systolic and diastolic function were achieved without reduction in arterial pressure. Accordingly, antifibrosis effects of HGF might attenuate the progress of cardiomyopathy.

Cardiac dysfunction of cardiomyopathic hamster is an inherited condition[27] caused by an autosomal recessive mutation in the gene for δ-sarcoglycan.[28] Although the physiological consequences of the genetic defect remain unclear, investigators have demonstrated that these animals display calcium handling abnormalities,[29] inhomogeneous capillary flow,[30] and microvascular spasm.[1] HGF might exert beneficial effects on the cardiovascular system via potentiation of angiogenesis[31] and vasodilation[32] against vascular spasm and antifibrosis action[5–7] against fibrosis subsequent to calcium overload and ischemia.

In this study, elevated levels of HGF might result in prevention of the progress of LV systolic dysfunction, which was measured by echocardiography. Moreover, HGF might reverse the impaired relaxation measured by E/A ratio and isovolumic relaxation time and impaired LV filling measured by deceleration time of E wave and deceleration rate of E

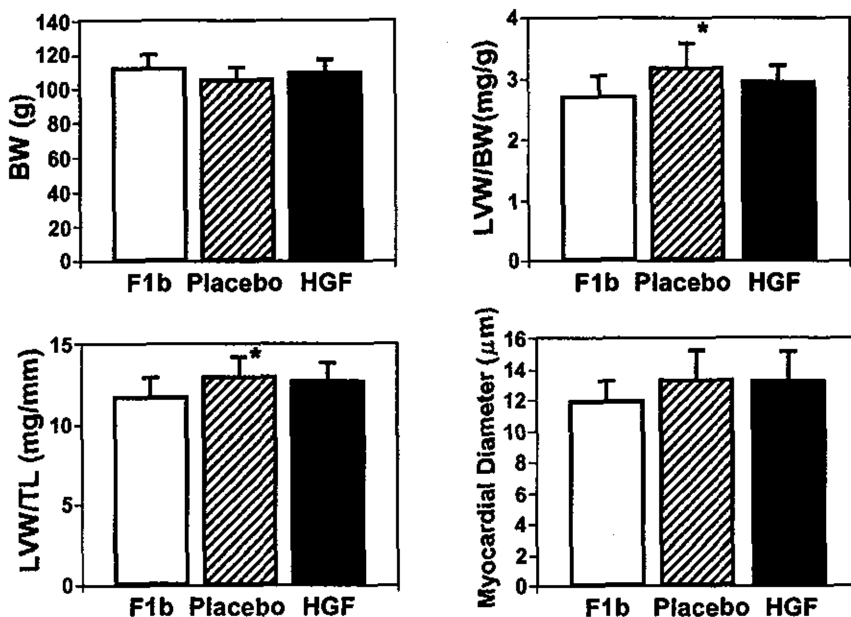

Figure 5. Pathological parameters on day 21 in each group. Data are expressed as mean±SD. BW indicates body weight; LVW, left ventricular weight, TL, tibialis length. *$P<0.05$ vs F1b.

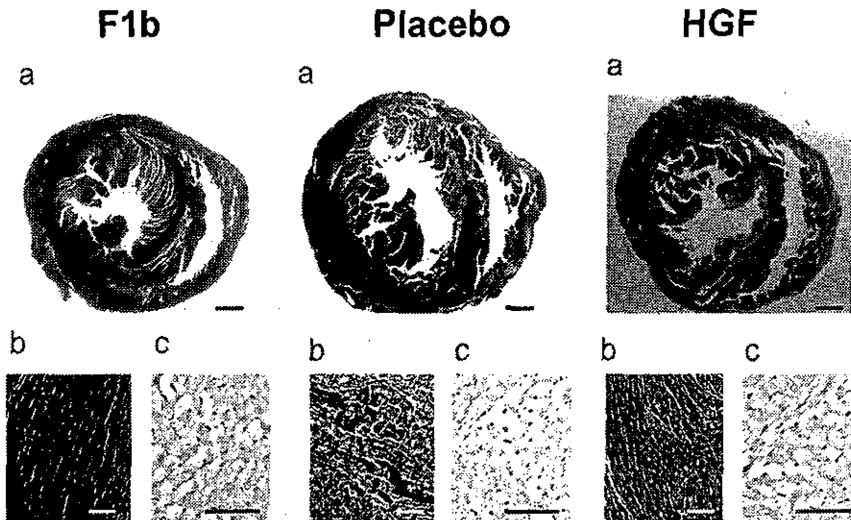

Figure 6. Representative histological images of myocardium in each study group. a, Macroscopic views of ventricular slice stained with Masson-Trichrome, black bar indicates 1 mm, inner diameter of F1b heart was 4.4 mm, placebo heart was 6.3 mm, and HGF heart was 4.8 mm. b, Microscopic (×200) views of myocardium stained with Masson-Trichrome, white bar indicates 25 μm. c, Microscopic (×400) views of myocardium stained with von Willebrand factor, black bar indicates 25 μm.

wave. Furthermore, HGF might reverse deteriorated hemodynamics. HGF might attenuate the increase in fibrosis and hydroxyproline and the decrease in capillary density in the myocardium of cardiomyopathy.

HGF is a potent activator of MMP-1, leading to collagen type 1 degradation and contributing to matrix restructuring before angiogenesis.[33,34] In studies of liver fibrosis,[35] increased HGF resulted in a 2-fold increase in interstitial MMP-1 activity and suppression of collagen deposition. These data were consistent with our results, which demonstrated a 2-fold increase in myocardial MMP-1 activity with increased HGF. The negative correlations between MMP-1 activity and fibrosis area and positive correlations between MMP-1 activity and capillary density suggest that increased HGF may exert its beneficial effects via stimulation of MMP-1.

The TO-2 cardiomyopathic hamster experiences rapid progression of heart failure with increasing age. Ryoke et al demonstrated that treatment of young TO-2 hamsters with growth hormone showed beneficial effects on cardiovascular function, whereas treatment of older TO-2 hamsters had little effect.[2] Growth hormone decreases collagen type I in the failed heart.[36] If this is the case, HGF might not have protective effects on the myocardium with advanced cardiomyopathy in older hamsters. Thus, the beneficial effects of exogenous HGF in our model might be dependent on a hamster's younger age or milder status of cardiomyopathy.

HGF decreased arterial pressure in rats.[32] SVR significantly decreased in the HGF-treated hamsters compared with the placebo hamsters in the present study. However, arterial pressure did not change at all. The capillary density in the transfected tibialis anterior muscles was greater in HGF hamsters than in placebo hamsters in the present study (1345 ± 95 versus 1163 ± 117 number/mm^2, $P<0.05$, n=5). However, the capillary density in other muscles such as gastrocnemius and quadriceps femoris muscles did not show differences. Differences in the density of HGF receptors

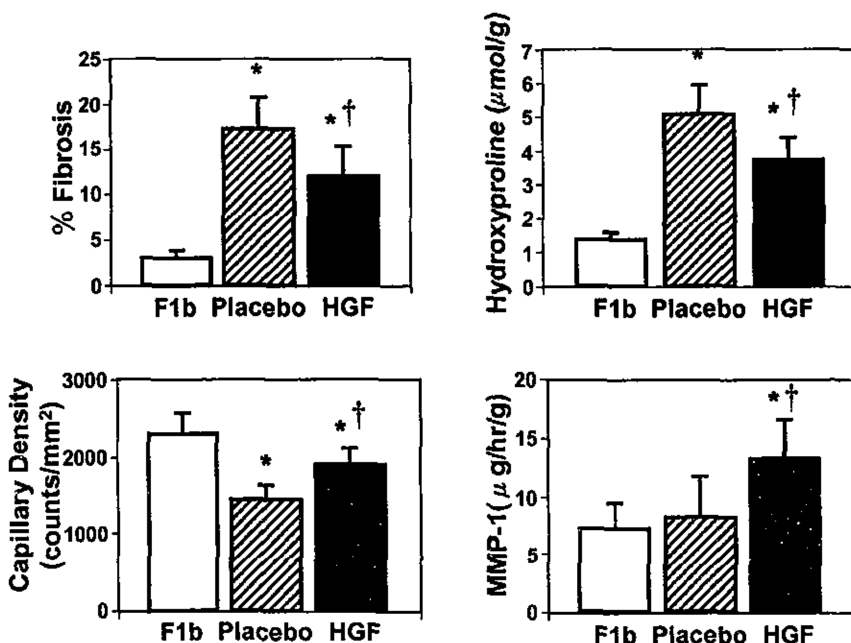

Figure 7. Histological and biochemical parameters of myocardium in each study group. Data are expressed as mean±SD. *$P<0.05$ vs F1b. †$P<0.05$ vs placebo.

between the heart and skeletal muscles or differences in response thresholds for systemic HGF protein might be attributable to the observed differences in the changes in capillary densities, and unchanged capillary density might explain the unchanged arterial pressure. Increment in cardiac output might be the primary cause of decrease in vascular resistance. The point of the present study was the improvement in cardiac systolic and diastolic function, and myocardial interstitial fibrosis without significant changes in blood pressure. Thus, antifibrotic action of HGF played an important role in the treatment of dilated cardiomyopathy.

Previous studies have demonstrated that the use of electroporation with a plasmid vector for interleukin (IL)-5 gene transfection resulted in a significantly elevated serum IL-5 level that persisted for at least 3 weeks.[13] Further, electroporation of the IL-10 gene into skeletal muscle using this vector resulted in the attenuation of the progression of autoimmune myocarditis and a decrease in mortality.[17] In the present study, we demonstrated that transfection of HGF gene into skeletal muscle resulted in systemic delivering of HGF protein and improved myocardial function and structure in animal model of idiopathic dilated cardiomyopathy.

Perspectives

Favorable effects of HGF on myocardial function and structure in the experimental cardiomyopathic heart should be used in the clinical situation. HGF gene and protein may be effective for the prevention of the progress of idiopathic dilated cardiomyopathy in humans and may decrease the number of candidates for heart transplantation. Systemic administration of HGF has risks for cancer proliferation or deterioration of diabetic retinopathy. Heart-specific local delivery of HGF is required for the future clinical application.

Acknowledgments

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Genetic analysis of 22 candidate genes for hypertension in the Japanese population

Naoharu Iwai, Naomi Tago, Naomi Yasui, Yoshihiro Kokubo, Nozomu Inamoto, Hitonobu Tomoike and Keisuke Shioji

Objective **We performed association studies between 118 single-nucleotide polymorphisms (SNPs) of 22 candidate genes (or gene family) and hypertension in a Japanese population.**

Design and participants **The study population consisted of 1880 subjects representing the general population in Japan, recruited from the Suita study. The candidate genes were selected based on their functions, including insulin resistance (*APM1, CD36, HSD11B1*), oxidative stress (*CYBA, GPX1, GSTMs*), steroid hormone (*ESR1, ESR2, HSD11B2*), renal functions (*PTGS2, KLK1, NPHS1, NPHS2, SGK, SLC12A1, PTGES*), and others related to cardiovascular physiology (*GJA4, NOS1, NTRK3, P2RX4, SPP1, ALDH2*).**

Results **Multiple logistic analyses, with age and body mass index as covariates, indicated that 13 SNPs (eight genes), six SNPs (four genes) and 11 SNPs (four genes) were associated with hypertension ($P<0.05$) in the total, male, and female populations, respectively. *PTGS2* seems to be a promising candidate gene for hypertension in men. *GSTM3* and *SLC12A1* seem to be promising candidate genes for hypertension in women. Especially, a polymorphism in *SLC12A1* was significantly associated with hypertension in women even after correction by the Bonferroni method (corrected $P=0.0236$). Multiple logistic analyses, with age and body mass index as covariates, indicated that the prevalence of hypertension in females was significantly higher in subjects with the CC genotype than in those with the TT + TC genotypes ($P<0.0001$, odds ratio = 1.967, 95% confidence interval = 1.430–2.712).**

Conclusion **Although the present results should be replicated in other study populations for confirmation, the present results suggest that *SLC12A1* may contribute to hypertension in Japanese women. *J Hypertens* 22: 1126–1126 © 2004 Lippincott Williams & Wilkins.**

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Introduction

Interactions between genetic and environmental factors are thought to play key roles in the pathogenesis of hypertension. The use of association studies in large epidemiological cohorts with a large number of single-nucleotide polymorphisms (SNPs) throughout a single gene or throughout the entire genome is expected to be a new strategy for identifying genes that contribute to hypertension [1,2].

However, recent genome-wide linkage scans for hypertension have found limited evidence of genes that determined hypertension [3,4]. This genome-wide scan strategy is largely dependent on the assumption that common diseases are explained by a combination of common disease alleles. In the current debate about alleles for common diseases, such as hypertension and diabetes, there are two extreme hypotheses: the common disease/common allele hypothesis and the common disease/rare allele hypothesis [5,6]. The failure of the genome-wide scan strategy seems to support the common disease/rare allele hypothesis [7].

On the other hand, we have confirmed that the ALDH2 genotype significantly influenced the blood pressure level in Japanese men by influencing alcohol intake [8]. The rare allele frequency of *ALDH2* is about 0.3, and this may be an example of the common disease/common allele hypothesis. It is possible that the blood pressure level is influenced mainly by a large number of younger and more population-specific alleles. We think it is still worth pursuing the candidate gene approach based on the common disease/common allele hypothesis in Japanese, who comprise a relatively homogeneous population.

Another possible reason for the failure of genome-wide scans for hypertension may be a lack of statistical

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power. The odds for hypertension alleles might be less than expected, and the number of subjects needed for clear detection of an association might be much greater than expected. If so, just a single study that considers even several thousand subjects might not be enough to give a firm conclusion and, as advocated by several researchers, meta-analyses might be required [9].

Moreover, the recent development of high-throughput technology in genotyping enables us to determine hundreds of SNP genotypes in thousands of subjects in a reasonable time, and has led to the problem of multiple testing. Since Bonferroni correction seems to be impractical, we should alternatively perform repeated testing in other study populations. Thus, any single study that considers just a few thousand subjects may not be enough for a clear conclusion and should be viewed as providing only tentative results.

In the present study, we performed association studies between 118 SNPs of 22 candidate genes (or gene family) and hypertension. We found that several SNPs were significantly associated with hypertension ($P < 0.05$), and one SNP in SLC12A1 was significantly associated with hypertension in females even after Bonferroni correction. We hope the present results may be useful in other genetic epidemiological studies on hypertension.

Materials and methods

Study population

The selection criteria and design of the Suita Study have been described previously [10,11]. The sample consisted of 14 200 men and women (30–79 years of age), stratified by gender and 10-year age groups, who had been randomly selected from the municipal population registry. They were all invited, by letter, to attend regular cycles of follow-up examination (every 2 years). We routinely check up 10–15 participants per day. DNA from leukocytes was collected from participants who visited the National Cardiovascular Center between April 2002 and February 2003. All of the participants were Japanese, and only those who gave written informed consent for genetic analyses of cardiovascular diseases were included. The genotype was determined in 1880 consecutive participants. The ethics committee of the National Cardiovascular Center approved the study protocol.

Blood pressure was measured after 10 min of rest in a sitting position. Systolic blood pressure and diastolic blood pressure values were the means of the two physician-obtained measurements (recorded > 3 min apart). Physicians obtained detailed personal medical information (past history, present illness, medication, etc.) directly from the participants of the Suita Study.

The characteristics of the subjects analyzed in the present study are summarized in Table 1. The diagnosis of hypertension was based on blood pressure measurement (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg), the current use of antihypertensive medication, or the diagnosis of family doctors.

Selection of candidate genes and polymorphisms

The selection of candidate genes was based on their physiological functions, as summarized in Table 2 [12–37]. Polymorphisms of the 11 genes were selected from the JSNP database [38] (http://snp.ims.u-tokyo.ac.jp/). We tried to select polymorphisms to cover an entire gene at an even spacing of 10–20 kb. We screened for polymorphisms of the genes indicated in bold in Table 2 (11 genes) by sequencing the promoter and all of the exons in 48–96 subjects. We identified 125 polymorphisms in these 11 genes. The sequence and polymorphisms data are available upon request. The sequences of polymorphisms genotyped in the present study but not described in the JSNP and NCBI databases are presented in Table 3.

In a single gene, the degree of linkage disequilibrium among SNPs with frequency > 0.15 was calculated using the SNPAlyze statistical analysis package (Dynacom, Yokohama, Kanagawa, Japan). When the R-square value of linkage disequilibrium was more than 0.25, the SNPs were categorized into a single group. At least one representative SNP from each group was included in the genotyping. Generally, rare SNPs (< 0.15) were not included in the analysis, except for missense, promoter,

Table 1 Characteristics of the study population

	Male	Female	P
n	867	1013	
Age (years)	66.31 (11.05)	63.33 (11.02)	< 0.0001
Body mass index (kg/m^2)	23.22 (0.10)	22.30 (0.10)	< 0.0001
Systolic blood pressure (mmHg)	134.8 (19.4)	128.1 (19.7)	< 0.0001
Diastolic blood pressure (mmHg)	79.7 (10.7)	76.6 (9.8)	< 0.0001
Prevalence of hypertension (%)	45.9	37.2	< 0.0001
Prevalence of antihypertensive treatment (%)	27.3	22.7	0.0207
Prevalence of subjects with myocardial infarction (%)	2.1	0.5	0.0016
Prevalence of subjects with cerebrovascular accident (%)	3.6	1.4	0.0018

[a]Data presented as mean (standard deviation).

Table 2 Gene functions for candidate genes

Function	Gene	Reference
Insulin resistance	APM1 (adiponectin)	[12]
	CD36	[13]
	HSD11B1	[14]
Oxidative stress	**CYBA** (p22-PHOX)	[15]
	GPX1 (glutathione peroxidase 1)	[16,17]
	GSTMs (glutathione S-transferase)	[16,18]
Steroid	**ESR1** (estrogen receptor alfa)	[19]
	ESR2 (estrogen receptor beta)	[20,21]
	HSD11B2	[22]
Renal functions	**PTGS2** (cyclooxygenase2)	[23]
	KLK1 (kallikrein1)	[24]
	NPHS1 (nephrin)	[25]
	NPHS2 (podocin)	[26]
	SGK1	[27]
	SLC12A1 (NKCC2)	[28]
	PTGES	[29]
Miscellaneous	**GJA4** (connexin37)	[30,31]
	NOS1	[32]
	NTRK3	[33]
	P2RX4	[34]
	SPP1 (osteopontin)	[35,36]
	ALDH2	[37]

Polymorphisms in the genes in bold were screened by sequencing.

or possibly functional mutations. We determined the genotypes of 118 SNPs of the 22 candidate genes in 1880 subjects. Polymorphisms were determined by the TaqMan method. The number of undetermined samples due to experimental error was less than 30 (1.6%). The details of primers and probes will be provided on request.

Statistical analysis

Values are expressed as mean ± standard deviation. All statistical analyses were performed with the JMP statistical analysis package (SAS Institute, Inc., Cary, North Carolina, USA). Multiple logistic (presence or absence of hypertension) and regression (blood pressure value) analyses were performed to assess the contribution of each genotype to hypertension, with age and body mass index (BMI) as covariates. We included BMI as a covariate because our preliminary assessment indicated that none of the genotypes seemed to influence blood pressure levels by affecting BMI levels. The systolic blood pressure and diastolic blood pressure values of subjects with antihypertensive medication were corrected by simply adding 10 or 5 mmHg, respectively, as advocated by Cui *et al.* [39]. To prevent false-positives caused by multiple testing, the *P* value was corrected by the Bonferroni method. Statistical power was calculated by the Sample Power software package (SPSS Inc., Chicago, Illinois, USA).

Results

Tables 4 and 5 summarize the results of the association study. The SNP-ID indicates the SNP identification number in the JSNP database [38] (http://snp.ims.u-tokyo.ac.jp/) or the NCBI database. If the polymorphisms are not described in these databases, the sequences around the SNP are presented in Table 3. We determined the genotypes of 118 SNPs of the 22 candidate genes in 1880 subjects.

We performed multiple logistic analyses with age and BMI as covariates. We observed significant associations ($P < 0.05$) in 13 SNPs of eight genes (*APM1*, *PTGS2*, *CYBA*, *GSTMs*, *NOS1*, *SLC12A1*, *PTGES*, *ALDH2*) in the total population, in six SNPs of four genes (*APM1*, *PTGS2*, *NPHS1*, *ALDH2*) in the male population, and in 11 SNPs of four genes (*GSTMs*, *NOS1*, *SLC12A1*, *PTGES*) in the female population. The ALDH2 genotype appeared to affect blood pressure levels in men in

Table 3 Sequences of the polymorphisms not described in the database

SNP number[a]	Gene	Region	Sequence
14	CYBA	5′ region	atggggaataaaccagcatt[g/a]ctgcctccggcctccgccgt
15		5′ region	tctgagtgaccctggcacct[c/g]tcagggagtccaggtgtgcc
16		Exon 4(H > Y)	cctcccccaggggacagaag[t/c]acatgaccgccgtggtgaag
17		Exon 6(A > V)	cgtgtctagtgtgtcgggtc[a/g]tttccttgcatttcctcatt
19	ESR1	Intron 5	caaattcacagaaagctaag[g/a]ataactttctgctagacatt
23	ESR2	5′ region	catctttggagcctgtcttc[a/g]ttacctgtggacctcatctt
25		5′ region	aacgcgaaaggccttcccag[t/c]gacctcttgagagctgagaa
26		Intron 4	tgtggtgagtgcttgcttcc[c/t]ttcttattgaatatgggcct
28		Exon 8(A > V)	tgacagcagccggaagctgg[c/t]tcacttgctgaacgccgtga
30	GJA4	5′ region	caaggagtccatgggaattg[c/t]ttaatggcctctgattgtgt
95	SGK	5′ region	tggtaacttgtaactgcccg[c/t]tccgggcccagtcgccgtgc
98	SLC12A1	5′ region	gctactcagacaactggaaa[t/c]cacttaaatcgtttcttata
99		Exon 4b(G > C)	gtcttggtgtgattatcatc[c/g]gcttagccgtgacagtgact
100		Intron 6	tcaaaaatgatattatcaac[a/t]gtggctggtcaggtcctgaa
101		Exon 9′	ggttttgagggatgaacagg[a/g]gtttgccgacaggacaaaga
104		Exon 17.1(A > V)	ggagatgaacagtggcatgg[c/t]gaaaaaacagg[c/t]ctggctt
105		Exon 17.2(A > V)	tggcatgg[c/t]gaaaaaacagg[c/t]ctggcttataaagaacaaaa
107		Intron 21	cttccatttagatatactc[a/t]ttgtgtcataaatttattct
109		3′ region	gatatgcaaacctctggaga[g/t]gatcctaccagattctacat
110	PTGES	5′ region	ctgaagagtcagttgatagg[t/a]ctttcggggagatcttgtga
111		5′ region	ggatagtcaagtgatgctca[g/a]gaagtcagtggagccacgtg
112		5′ region	aggctgagtgtgggggcgca[t/c]ggcgtggttctcatgcccac
113		Exon 2	ataaaagcagagacaggggg[g/a]ccttttcatggtgagctacc
115	SPP1	5′ region	gtaaaggacagaggcaagtt[t/c] tctgaactccttgcaggctt

Table 4 Associations between single-nucleotide polymorphisms (SNPs) and hypertension

SNP number	Gene	Region	SNP identification	MIAF	Total	Male	Female
1	APM1	5′ region	IMS-JST118935	0.4757	0.1678	0.8658	0.1298
2		5′ region	IMS-JST046297	0.4663	0.4888	0.9853	0.3781
3		Intron 1	IMS-JST013728	0.2700	0.6051	0.5559	0.3983
4		Intron 1	IMS-JST129672	0.2899	0.0286	0.0010	0.4333
5	CD36	5′ region	IMS-JST005702	0.2908	0.8586	0.9800	0.5972
6		Exon 3(P > S)	IMS-JST119289	0.0391	0.3938	0.9479	0.2803
7		Intron 4	IMS-JST088751	0.2071	0.1928	0.2112	0.2479
8		Intron 7	IMS-JST166447	0.4318	0.3157	0.3659	0.7371
9		Intron 9	IMS-JST184511	0.1636	0.9527	0.7744	0.9048
10	PTGS2	5′ region	rs689466	0.4490	0.0100	0.0225	0.1894
11		5′ region	rs4987005	0.0163	0.0669	0.0297	0.6340
12		Intron 6	rs20431	0.0173	0.0508	0.0250	0.6386
13		Exon 10(G > R)	rs3218625	0.0143	0.1894	0.0796	0.8317
14	CYBA	5′ region	gcatt[g/a]ctgcc	0.4569	0.2414	0.4197	0.5102
15		5′ region	cacct[c/g]tcagg	0.2543	0.7713	0.8325	0.2820
16		Exon 4(H > Y)	agaag[t/c]acatg	0.0861	0.0388	0.1111	0.1590
17		Exon 6(A > V)	gggtc[a/g]tttcc	0.1709	0.2647	0.7147	0.1414
18	ESR1	Exon 1	rs2077647	0.3973	0.2810	0.3706	0.5837
19		Intron 5	ctaag[g/a]ataac	0.2723	0.1361	0.5365	0.1422
20		Intron 6	rs2207396	0.1833	0.5752	0.6552	0.6206
21		Intron 6	rs974276	0.3174	0.6657	0.9274	0.5322
22		Exon 8	rs2228480	0.1598	0.5641	0.2420	0.4508
23	ESR2	5′ region	tcttc[a/g]ttacc	0.0040	0.6038	0.8164	0.3677
24		5′ region	rs1271572	0.3951	0.8373	0.8494	0.5775
25		5′ region	cccag[t/c]gacct	0.0707	0.9105	0.9455	0.5383
26		Intron 4	cttcc[c/t]ttctt	0.0676	0.1458	0.5673	0.2649
27		Exon 6	rs1256049	0.2859	0.6447	0.6674	0.7753
28		Exon 8(A > V)	gctgg[c/t]tcact	0.0040	0.6656	0.2664	0.4598
29		Intron 8	rs944650	0.3535	0.2212	0.2979	0.5853
30	GJA4	5′ region	aattg[c/t]ttaat	0.0784	0.5993	0.1555	0.3101
31		Exon 1	IMS-JST084084	0.0392	0.4192	0.8472	0.3744
32		Exon 2(P > S)	rs1764391	0.0392	0.8027	0.9137	0.9074
33		Exon 2	IMS-JST084085	0.2465	0.4616	0.2126	0.9285
34		Exon 2	IMS-JST084087	0.4922	0.5470	0.4609	0.8040
35		Exon 2	IMS-JST084089	0.0675	0.8453	0.8079	0.8946
36		3′ UTR	IMS-JST181134	0.3159	0.5127	0.8310	0.2432
37	GPX1	5′ region	rs3811699	0.0659	0.4431	0.1543	0.9011
38		3′ UTR	rs1050614	0.0607	0.3619	0.2330	0.6698
39	GSTMs	GSTM2	IMS-JST009839	0.2534	0.6062	0.6633	0.7436
40		GSTM2	ssj0002173	0.2106	0.5252	0.5042	0.8002
41		GSTM1	ssj0002162	0.0171	0.6937	0.7608	0.7656
42		GSTM5	IMS-JST107445	0.3031	0.0936	0.7949	0.0522
43		GSTM5	IMS-JST051979	0.3092	0.0635	0.6336	0.0425
44		GSTM5	IMS-JST123133	0.0171	0.9372	0.8146	0.8098
45		GSTM3(I > V)	rs7483	0.2336	0.0145	0.5206	0.0094
46		GSTM3	ssj0004679	0.0803	0.5347	0.6759	0.2162
47		GSTM3	rs4970777	0.1808	0.1850	0.4892	0.1974
48		GSTM3	rs4970737	0.3030	0.0092	0.3869	0.0063
49		GSTM3	IMS-JST030783	0.2988	0.1031	0.5882	0.1329
50	HSD11B1	5′ region	IMS-JST106455	0.3091	0.3975	0.1451	0.0919
51		Intron 3	IMS-JST017378	0.4883	0.8315	0.7118	0.3588
52		3′ region	IMS-JST119354	0.1517	0.1049	0.0518	0.9412
53	HSD11B2	5′ region	IMS-JST066125	0.0431	0.7035	0.8597	0.4278
54		5′ region	IMS-JST141629	0.0230	0.9417	0.7118	0.9076
55		3′ region	IMS-JST026559	0.0067	0.6197	0.9149	0.4747
56		3′ region	IMS-JST095004	0.0966	0.7024	0.1532	0.4411
57	KLK1	5′ region	IMS-JST096981	0.2993	0.2143	0.0974	0.4764
58		5′ region	IMS-JST096980	0.4115	0.3145	0.5122	0.3937
59		Exon 3(K > E)	IMS-JST179917	0.5000	0.4179	0.4582	0.7785
60		Exon 4	IMS-JST179923	0.2104	0.1876	0.6797	0.1727
61	NOS1	Intron 1	IMS-JST138598	0.4103	0.3265	0.2771	0.6057
62		Exon 2	IMS-JST092495	0.0111	0.7115	0.7488	0.7506
63		Intron 2	IMS-JST138590	0.1059	0.2894	0.1066	0.4500
64		Intron 9	IMS-JST046030	0.2165	0.1796	0.3236	0.2542
65		Intron 11	IMS-JST092494	0.3813	0.7765	0.4384	0.0560
66		Exon 18(H > E)	IMS-JST044454	0.4745	0.0845	0.3591	0.0918
67		Intron 21	IMS-JST092492	0.4495	0.0351	0.6571	0.0207
68		Exon 22(D > E)	IMS-JST092489	0.3343	0.2898	0.2769	0.2173
69		Intron 26	IMS-JST067032	0.0950	0.0851	0.1808	0.4326
70		Exon 29	IMS-JST092487	0.3468	0.1891	0.3431	0.0397
71		Exon 29	IMS-JST092488	0.1577	0.1713	0.5560	0.2463
72	NTRK3	Intron 2	IMS-JST060131	0.1051	0.1434	0.0536	0.1955
73		Intron 5	IMS-JST003765	0.4882	0.0896	0.3267	0.2779
74		Intron 7	IMS-JST027105	0.3334	0.3105	0.2451	0.9008

Table 4 (*continued*)

SNP number	Gene	Region	SNP identification	MIAF	Total	Male	Female
75		Intron 7	IMS-JST074565	0.0200	0.2305	0.6732	0.1880
76		Intron 9	IMS-JST074567	0.1170	0.8780	0.7848	0.6653
77		Intron 10	IMS-JST003766	0.1554	0.7835	0.9756	0.7279
78		3′ region	IMS-JST162236	0.0200	0.8864	0.9709	0.9492
79	NPHS1	5′ region	IMS-JST000540	0.3027	0.5011	0.6841	0.4859
80		Exon 17	IMS-JST000542	0.0902	0.8533	0.2534	0.6816
81		Intron 23	IMS-JST033886	0.2098	0.4624	0.8432	0.3298
82		Exon 26	IMS-JST006746	0.3489	0.9327	0.7133	0.8110
83		3′ region	IMS-JST006747	0.1336	0.2724	0.0335	0.0859
84	NPHS2	5′ region	IMS-JST173815	0.0290	0.9180	0.4439	0.3013
85		Intron 1	IMS-JST119740	0.4057	0.6195	0.7570	0.7107
86		Exon 2	IMS-JST084167	0.1929	0.8459	0.8245	0.9823
87		Intron 6	IMS-JST070550	0.4510	0.3169	0.3703	0.6305
88		Exon 8	IMS-JST070547	0.4635	0.0996	0.7771	0.0713
89		3′ region	IMS-JST070542	0.0398	0.5084	0.3005	0.9683
90	P2RX4	5′ region	IMS-JST067157	0.3249	0.5864	0.6234	0.9197
91		Exon 2	IMS-JST060855	0.0030	0.8627	0.7347	0.6311
92		Exon 5	IMS-JST103166	0.3270	0.6228	0.8004	0.8445
93		Exon 7(S > G)	IMS-JST060854	0.3404	0.2644	0.1926	0.3774
94		Intron 10	IMS-JST006649	0.3093	0.5522	0.1436	0.9814
95	SGK1	5′ region	gcccg[c/t]tccgg	0.4117	0.4923	0.5774	0.7950
96		5′ region	rs1743963	0.1349	0.4554	0.6560	0.3670
97		Exon 8	rs1057293	0.1874	0.2368	0.5027	0.4345
98	SLC12A1	5′ region	ggaaa[t/c]cactt	0.4153	0.0943	0.1137	0.3442
99		Exon 4b(G > C)	tcatc[c/g]gctta	0.0004	0.7937	0.7859	0.9902
100		Intron 6	tcaac[a/t]gtggc	0.0197	0.4177	0.7791	0.2173
101		Exon 9′	acagg[a/g]gtttg	0.1090	0.1936	0.3161	0.2212
102		Intron 12	IMS-JST027033	0.3959	0.0035	0.1659	0.0062
103		Intron 14	IMS-JST043660	0.4727	0.0016	0.5018	0.0001
104		Exon 17.1(A > V)	catgg[c/t]gaaaa	0.0182	0.9617	0.8512	0.8230
105		Exon 17.2(A > V)	acagg[c/t]ctggc	0.0570	0.4996	0.2428	0.7820
106		Intron 17	rs1484551	0.0339	0.8743	0.9643	0.9351
107		Intron 21	tactc[t/a]ttgtg	0.4356	0.0026	0.6045	0.0004
108		Intron 24	IMS-JST043662	0.4619	0.0017	0.1261	0.0103
109		3′ region	ggaga[g/t]gatcc	0.1768	0.0285	0.5875	0.0034
110	PTGES	5′ region	atagg[t/a]ctttc	0.0155	0.0304	0.3226	0.0191
111		5′ region	gctca[g/a]gaagt	0.0514	0.8174	0.8930	0.8984
112		5′ region	gcgca[t/c]ggcgt	0.1658	0.7751	0.6527	0.9394
113		Exon 2	ggggg[g/a]ccttt	0.2130	0.5208	0.6257	0.3385
114		Exon 3	rs2302821	0.4263	0.3687	0.2954	0.8111
115	SPP1	5′ region	rs2853744	0.2317	0.3138	0.3624	0.7890
116		5′ region	aagtt[t/c]tctga	0.4199	0.8768	0.3438	0.5640
117		Exon 6	rs1126616	0.3353	0.3424	0.2027	0.6750
118	ALDH2	Exon 12(E > K)	rs671	0.2832	0.0476	0.0160	0.6085

MIAF; minor allele frequency. SNP identification was obtained from the JSNP home page (http://snp.ims.u-tokyo.ac.jp/) for 'IMS-JST xxxxxx' or 'ss xxxxxx', or from the NCBI home page (http://www.ncbi.nlm.nih.gov/) for 'rs xxxxxx'. Polymorphisms not described in these databases are presented in Table 3. *P* values were obtained by logistic analysis with age and body

the present study, and this confirmed our previous observation (the present sample was collected in 2002, and previous samples were collected in 1996–1998).

Three SNPs of *PTGS2* were significantly associated with hypertension in men (Tables 4 and 5). Three SNPs of *GSTM3* were significantly associated with hypertension in women (Tables 4 and 5).

The most striking associations were observed between *SLC12A1* SNPs and blood pressure levels in women. Logistic analysis indicated that the genotype of *SLC12A1*, IMS-JST043660, predicted the presence of hypertension in females ($P = 0.0001$ with age and BMI as covariates, and $P = 0.0002$ with only age as a covariate). Moreover, the genotype of *SLC12A1*, IMS-JST043660, was significantly associated with hypertension in females even after correction by the Bonferroni method ($P = 0.0001 \times 118$ (SNP number) $\times$ 2 (gender) $= 0.0236$).

The effects of this genotype on other phenotypic variables are presented in Table 6. Since the distortion of pressure recordings by antihypertensive treatment has been suggested to obscure underlying genetic effects [39], we added 10 and 5 mmHg, respectively, to systolic blood pressure and diastolic blood pressure values of treated subjects for correction.

The CC genotype had higher residuals of systolic blood pressure after adjusting for age and BMI ($P = 0.0173$). Multiple logistic analyses, including age and BMI as covariates, indicated that female subjects with the CC genotype had significantly higher prevalence of hypertension ($P < 0.0001$, odds ratio = 1.967, 95% confidence interval = 1.430–2.712) and antihypertensive medica-

Table 5 Candidate single-nucleotide polymorphisms (SNPs) and frequency of hypertension

SNP number	Gene	Gender	Major	Hetero	Minor	*P*
4	APM1	Male	195/446 (43.72)	176/342 (51.46)	26/76 (34.21)	0.0010
		Female	197/517 (38.10)	149/410 (36.34)	28/82 (34.15)	0.4333
10	PTGS2	Male	109/262 (41.60)	188/420 (44.76)	97/177 (54.80)	0.0225
		Female	110/312 (35.26)	179/485 (36.91)	81/107 (75.70)	0.1894
11		Male	376/833 (45.14)	18/27 (66.67)		0.0297
		Female	360/975 (36.92)	13/34 (38.24)		0.6340
12		Male	377/832 (45.31)	20/31 (64.52)		0.0250
		Female	359/971 (36.97)	13/34 (38.24)		0.6386
16	CYBA	Male	319/697 (45.77)	71/152 (46.71)	6/7 (85.71)	0.1111
		Female	312/862 (36.19)	52/126 (41.27)	8/14 (57.14)	0.1590
43	GSTMs	Male	190/408 (46.57)	170/372 (45.70)	38/86 (44.19)	0.6336
		Female	199/492 (40.45)	143/420 (34.05)	32/98 (32.65)	0.0425
45		Male	248/522(47.51)	121/279 (43.37)	25/58 (43.10)	0.5206
		Female	237/586 (40.44)	114/353 (32.29)	21/60 (35.0)	0.0094
48		Male	197/417 (47.24)	160/355 (45.07)	35/83 (42.17)	0.3869
		Female	202/493 (40.97)	130/401 (32.42)	33/99 (33.33)	0.0063
67	NOS1	Male	125/269 (46.47)	202/428 (47.20)	67/160 (41.88)	0.6571
		Female	105/293 (35.84)	197/485 (40.62)	105/293 (35.84)	0.0207
70		Male	178/368 (48.37)	167/385 (43.38)	44/98 (44.90)	0.3431
		Female	149/414 (35.99)	183/456 (40.13)	34/120 (28.33)	0.0397
83	NPHS1	Male	280/641 (43.68)	103/203 (50.74)	11/16 (68.75)	0.0335
		Female	284/758 (37.47)	85/227 (37.44)	2/18 (11.11)	0.0859
110	PTGES	Male	376/811 (46.36)	12/34 (35.29)	1/1 (100)	0.3226
		Female	364/966 (37.68)	4/22 (18.18)		0.0191
118	ALDH2	Male	220/443 (49.66)	151/348 (43.39)	22/67 (32.84)	0.0160
		Female	193/520 (37.12)	149/381 (39.11)	29/93 (31.18)	0.6085

The frequencies of hypertensive subjects according to genotypes are described as number of hypertensive subjects/total number of the subjects (percentage of hypertensive subjects). *P* values were obtained by logistic analyses with age and body mass index as covariates.

Table 6 Phenotype and genotype relationship in *SLC12A1*

	Male				Female			
	TT	TC	CC	*P*	TT	TC	CC	*P*
n	191	426	250		222	525	266	
Age (years)	66.9 (11.0)	66.3 (11.1)	65.8 (11.1)	0.5963	63.2 (11.3)	63.8 (11.2)	62.5 (10.4)	0.2566
Body mass index (kg/m^2)	23.3 (2.9)	23.2 (3.2)	23.2 (2.7)	0.9315	22.3 (3.1)	22.3 (3.2)	22.3 (3.2)	0.9887
Systolic blood pressure (mmHg)	132.2 (18.2)	132.0 (19.7)	131.1 (19.9)	0.8129	126.6 (18.9)	127.9 (18.9)	129.6 (21.8)	0.2274
CSBP (mmHg)	134.5 (19.9)	134.9 (21.8)	133.9 (22.2)	0.8427	128.2 (20.6)	130.3 (20.9)	132.2 (23.7)	0.1341
ResCSBP (mmHg)	−0.5 (18.5)	0.4 (20.1)	−0.3 (20.1)	0.8243	−2.0 (18.6)	−0.5 (17.9)	2.6 (20.2)	0.0173
Diastolic blood pressure (mmHg)	79.5 (11.1)	79.8 (10.7)	79.7 (10.4)	0.829	75.6 (90.8)	76.6 (9.3)	77.4 (10.7)	0.1154
CDBP (mmHg)	80.6 (11.6)	81.2 (11.5)	81.1 (11.0)	0.7409	76.4 (10.3)	77.8 (10.0)	78.7 (11.4)	0.0534
ResCDBP (mmHg)	−0.5 (10.8)	0.2 (11.0)	0.0 (10.6)	0.7539	−1.3 (10.0)	0.0 (9.8)	1.1 (11.0)	0.0296
Prevalence of hypertension (%)	43.5	46.7	46.4	0.7409	30.6	36	45.1	0.0032
Prevalence of antihypertensive treatment (%)	23.6	28.9	27.6	0.3822	17.1	23.4	25.9	0.0521

CSBP; systolic blood pressure values of subjects with antihypertensive treatment were corrected by adding 10 mmHg, CDBP; diastolic blood pressure values of treated subjects were corrected by adding 5 mmHg, ResCSBP; residuals of CSBP after adjusting for age and body mass index; ResCDBP; residuals of CDBP after adjusting for age and body mass index.

tion ($P = 0.0224$, odds ratio = 1.512, 95% confidence interval = 1.058–2.153) than those with the TT + TC genotypes. The sample power of the association between this polymorphism and hypertension was calculated as 86% (alpha = 0.05).

We performed haplotype analyses in *SLC12A1*. However, the most significant association was observed with the single genotype IMS-JSNP043660. This polymorphism is located in the polypyrimidine-rich tract near a splicing acceptor site (not shown).

Discussion

In the present study, we performed a large-scale association analysis between 118 SNPs of 22 candidate genes and hypertension. We found that several polymorphisms significantly affected the blood pressure level with a classical criterion of $P < 0.05$. However, when we applied the Bonferroni method to correct *P* values, only the *SLC12A1* polymorphism significantly influenced the blood pressure level in women ($P = 0.0236$, Bonferroni). This genotype did not have a prominent effect on blood pressure values. This may have been due to the influence of antihypertensive treatment, since the genotype associated with hypertension was also significantly associated with a higher prevalence of antihypertensive treatment.

There may be no accurate way to correct treated blood pressure values. Thus, we mainly analyzed the data using a categorical variable (hypertension versus normotension) in the present study. We adopted Cui *et al.*'s proposal by simply adding 10/5 mmHg [39] because their work is the only published paper that deals directly with the problem of how to correct treated blood pressure values in genetic association studies. We included these corrected values in Table 6, but just for reference. We did not highlight these corrected values in the present study.

SLC12A1 is one of the genes responsible for antenatal Bartter syndrome, and its product has Na–K–2Cl co-transporter activity in the thick ascending limb of the loop of Henle [28]. Thus, *SLC12A1* may contribute to hypertension in women. It remains to be determined why this gene does not contribute to hypertension in men. A gender difference has been reported in salt sensitivity. For example, low-renin hypertension has been recently reported to be a significant predictor of systolic sodium sensitivity in females but not in males [40].

Although we screened all of the exons and intron–exon boundaries, we were unable to find strongly convincing variations in linkage disequilibrium with IMS-JST043660. This polymorphism was located in the polypyrimidine-rich tract in an intron, which might affect the mRNA level by influencing the splicing efficiency, and therefore may be functional in itself. Whether this polymorphism is a responsible functional variation or just in linkage disequilibrium with other important variations remains to be clarified. We did not sequence intronic regions because we are currently unable to clarify the biological significance of polymorphisms in these regions. However, it is possible that intronic variation is important: a polymorphism might confer a cryptic exon or a large deletion/insertion might alter the expression level of a transcript.

Although several SNPs with a classical criterion of $P < 0.05$ were excluded by the Bonferroni correction, we cannot conclude that these SNPs had no influence on the blood pressure level. We cannot tell whether these associations ($P < 0.05$ by the classical criteria) are true or false associations. We should perform additional association studies for these polymorphisms in other study populations before we reach final conclusions. Polymorphisms that confer a modest or slight risk of hypertension will be difficult to detect and tremendously large association studies may be necessary to obtain highly significant P values.

One of the striking features of the present study was the difference between men and women. This inconsistency may reflect a lack of statistical power due to the small sample size (1880 subjects may not be enough) or may reflect physiological gender differences. Again, to solve this problem, we should perform additional association studies in other study populations or identify intermediate phenotypes that explain this gender difference.

Since the Suita Study principally involves general health check-ups, it might be difficult to conduct more specific research-oriented laboratory and physiological tests. This drawback of the present epidemiological study should perhaps be offset by patient-oriented clinical studies that should follow re-confirmation of the validity of the present candidate genes in other study populations.

The mean age of the present study population was 66.3 years in males and 63.3 years in females, which are relatively old ages. Age has been reported to strongly affect the results of association studies. For example, the association of the Trp allele of the alpha-adducin gene and blood pressure was more evident at an older age, possibly due to the reduced efficiency of compensatory mechanisms [41,42]. On the other hand, the influence of beta2-adrenergic receptor polymorphism was more evident in younger individuals, presumably due to an age-related decline in beta2-adrenergic receptor-mediated activity [43]. Thus, association studies in younger populations might identify different sets of susceptibility genes.

In *SLC12A1*, we found by chance the A508T mutation, which has been reported to be one of the mutations responsible for the antenatal Bartter syndrome [44]. TaqMan analysis indicated that this mutation occurred in only one person, who had relatively low blood pressure, among 1880 subjects. In the sequencing analysis of candidate genes, we found several rare SNPs, some of which are missense mutations. Since these rare SNPs occur at a very low frequency, we could not confirm whether these SNPs might influence the blood pressure level due to the sample size in the present study. It is highly likely that we overlooked other rare SNPs with potentially important functions, since we only screened 48–96 subjects. If hypertension cannot be explained by common alleles but rather by rare alleles, we should change our strategy, sequence several hundred or even thousands of subjects, catalogue rare alleles, and perform an association study with a very large sample size.

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特集 ✚ 救急医療へのIT活用

循環器救急とモバイルテレメディシン

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要旨：高齢化社会を迎え、要介護状態の主因である心筋梗塞や脳卒中といった循環器救急医療の充実が期待されている。我々は、わが国が得意とする情報通信技術を活用したモバイルテレメディシンにより、病院前救護や救急搬送を充実すべく実証実験を続けている。

高齢化社会を迎え、要介護状態の主因である心筋梗塞や脳卒中に対する循環器疾患対策の重要性が増している。急性心筋梗塞（AMI）の致命率は高く、米国では死因の第1位といわれる。わが国における人口10万人あたりの死亡数は年間50人程度といわれているが、今後生活習慣の変化に伴う増加が懸念されている。院内死亡率は大幅に改善したものの、死亡の半数は院外で発生している[1,2]。従って、循環器救急医療、特に搬送体制と病院前救護の充実が急務である[3,4]。

近年、情報機器や移動体通信の発達により、モバイルテレメディシンという新分野が急速に発展しつつある。商用移動体通信の開発・普及が進んでいる日本への期待は大きい。

循環器救急医療でのニーズ

(1)適切な搬送（優先順位に基づく搬送）

わが国の救急医療体制は、重症度に応じて第2次救急（4005施設）から第3次（147施設）へと移送するシステムとして整備されている。超急性期医療の再灌流療法は、脳卒中で発症後3時間以内、AMIでも早期に開始することが重要である。

近年、救急専用電話（ホットライン）配備により直接搬送可能な専門医療機関が増加しているが、第3次救急へのAMIの直接搬送率は10%に留まり、発症—搬入時間には改善の余地がある。国立循環器病センターの調査でも、発症—搬入時間は直接搬送88分に対し他院経由は228分で、院内死亡率への影響（6.1%対23.5%、$p<.001$）が示唆されている[1,5]。

米国心臓学会（AHA）の救急治療国際ガイドライン[6]では、院外における12誘導心電図の自動診断/伝送が強く勧告されている。01年度には、わが国の救急車5517台による急病搬送226万8259件のうち、38万6786件で心電図が記録されたものの、9413件（2.4%）が伝送されたに過ぎない（消防白書）。

モバイルテレメディシンはトリアージ（傷病者優先順位）とディスパッチを支援することで、適切な搬送への貢献が期待される。

(2)病院前救護とメディカルコントロール体制

急性心筋梗塞による死亡の半数は院外で発生している。院外死亡を減少させるためには、病院前救護体制の充実が必要である[3]。

救命救急士制度は91年に発足し、医師の指示のもとで心肺機能停止状態の傷病者に対して特定医療行為（気道確保、輸液路確保、除細動）を実施できるようになった。01年度には高度な応急処置件数は約4万件、うち除細

●**Summary**
Mobile Telemedicine for Cardiovascular Emergency
—Information Technologies for Prehospital Care and Facility Triage—
Although in-hospital mortality has dramatically improved, half of the patients with myocardial infarction die before reaching hospitals. Mobile telemedicine is expected to revolutionize medical practice including better triage and pre-hospital care.

動が約5000件実施されている。

03年4月から、救急救命士法施行規則の改正により包括指示下での除細動（いわゆる指示なし除細動）が可能となり、04年度以降は薬剤投与や気管内挿管も可能になることから、メディカルコントロール体制の整備が求められている。

メディカルコントロール体制は、われわれ日本人がものづくりで世界に誇る品質保証活動の一種で、Plan-Do-Check-Act の4段階からなる。研修による知識習得と救急医による事後検証票の確認を中心としたオフライン・メディカルコントロールに加え、常時指示体制を充実させるオンライン・メディカルコントロールを実現するために、生体情報や音声・画像をリアルタイムに伝送できるモバイルテレメディシンの実現が期待されている。[7,8]

技術開発進むモバイルテレメディシン

(1)社会基盤の整備が進む遠隔医療

遠隔医療（テレメディシン）とは、通信と情報処理技術により、医療従事者が距離や時間を超えて共同で診療・研究することであり、テレビ会議の利用、遠隔眼科学、遠隔放射線診断学、遠隔病理学、遠隔心理学などが研究されてきた。[9]

わが国でも、97年に無診察治療を禁止する医師法第20条の解釈通知が出され、遠隔医療が保険診療化されるなど、社会基盤の整備が進んでいる。[10]

モバイルテレメディシンは、移動体通信技術を活用した遠隔医療の新しい分野である（図1）。既に、米国NIHとメリーランド州立大学が脳卒中を対象としたシステムを作成し、試験運用している。[11,12]

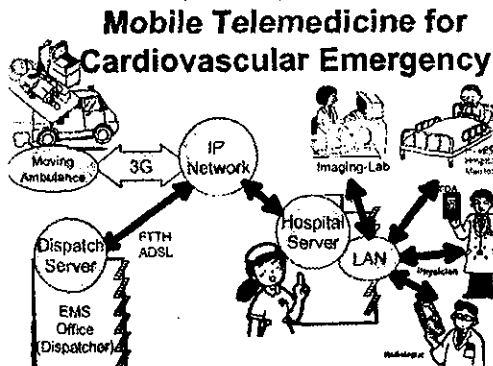

図1　モバイル・テレメディシンの構成

表1　移動体通信の発展と生体情報の伝送

移動体通信	バンド幅 bps	伝送可能な情報
第2世代（2G）	9.6K	バイタルサイン、心電図
PHS	64K	静止画、12誘導心電図
第3世代（3G）	64～384K	ベッドサイドモニタ、簡易動画
第4世代（4G）	10～100M	動画

(2)利用可能な移動体通信の現状と将来性

現在、救急車に装備されている消防・救急無線はアナログ方式（850～900MHz）で、秘匿性確保やデータ通信のためにデジタル方式（260MHz帯）への移行が予定されている。

近年、自動車電話・携帯電話の装備が進み、病院との連絡等に活用されている。第1世代（1G、アナログ）から、第2世代（2G、デジタル）を経て、第3世代（3G、高速デジタル）に移行しつつある。通信速度（表1）の面から3Gの商用化が進む日本への注目度は高い。人口比では96％以上をカバーする3G通信網だが、山間部等では不達地域も多い。

モバイルテレメディシンは、単一の通信技術に特化した「機器」ではなく、技術の進歩や地域格差に対応可能な「プラットフォーム」である。標準的インターネット技術であるTCP／IPを全面的に採用し、アプリケーション層、トランスポート層、インターネット層、ネットワーク・インターフェース層の4層に分離することで、高度道路通信システム（ITS）、衛星通信、4Gなどにも対応可能となっている。

(3)必要な生体情報の選別と符号化

心電図のアナログ伝送は、主として不整脈

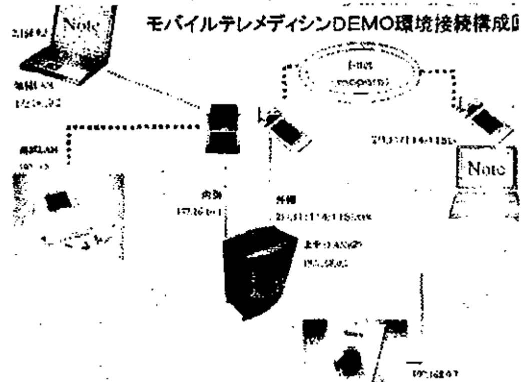

図2　プロトタイプの構成

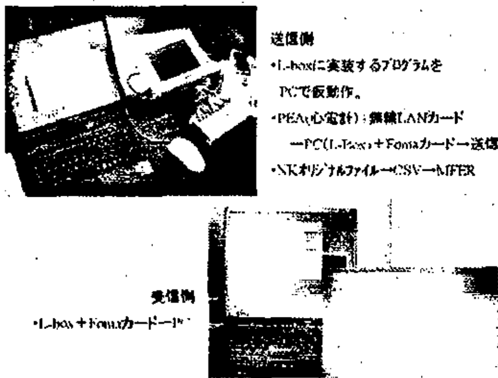

図3　プロトタイプを用いた実験

を対象とした解析技術とともに、遠隔医療の黎明期（1960年代後半）にNIHを中心に確立され、[9]世界中の病院で利用されている。12誘導への対応やデジタル化（符号化）は個々の医療機器レベルで対応されており、相互接続性やデータ互換性の問題を解決する必要がある。

血圧、脈拍、酸素飽和度等を監視するベッドサイド・モニタは、循環器救急における即時性の高い要求に応えるために有用で、動画や心電図と比べて情報量が少ないため、伝送は比較的容易である。

常時指示体制（オンライン・メディカルコントロール）の充実という面からは、音声通信のみの現状と比較した静止画または動画の持つ意義は大きい。[11,12]

例えば、外傷救急における患部の静止画伝送、脳卒中における動画伝送によりシンシナティ病院前脳卒中スケールやロサンゼルス病院前スクリーニング、将来的には、携帯型心臓超音波診断装置や体外診断薬による迅速生化学的診断等への対応も期待されている。

(4)データの圧縮・伸張技術

移動体通信では、通信速度や容量（バンド幅）に限りがあるため、圧縮・伸張技術（コーデック codec）が重要である。動画では、テレビ電話会議用のH320（ISDN）、H323（TCP／IP）、BS（衛星放送）やDVDで利用されているMPEG、Motion-JPEG等の規格が普及している。[9]

心電図伝送については、JPEGやH323等の非可逆圧縮では、歪みや遅延等の問題がある。今回われわれは、MFER（Medical Waveform Encoding Rule）（http://ecg.heart.or.jp）を採用した。MFERは日本の学会やメーカーが中心となって国際規格としてISO等に提案しているもので、HL7やDICOM等の電子診療録関連の規格とも整合性が高い。[13,14]

(5)プロトタイプの設計と製作

02年7月、「循環器救急におけるモバイルテレメディシン研究会」が設立され、産官学共同で、移動する救急車と病院をオンラインで結ぶシステムのプロトタイプが開発された。[3,4]救急車側には、12誘導心電計（日本光電）、ベッドサイド・モニタ（フクダ電子）、ネットワーク・カメラ（松下電器）を送受信384 Kbpsの3GであるFOMA（NTT DoCoMo）により接続し、TCP(UDP)／IPによるデータ伝送を行った。物理的・論理的変換装置には小型のLinuxマシン（NTTコムウェア）を利用した（図2）。病院側のクライアントには市販のPC（WindowsXP）を利用し、標準的インターネット・ブラウザ（Internet Explorer 6.0, Microsoft）を利用した（図3）。

心電図については、MFERの利用により標準12誘導心電図（10秒間、サンプリング周波数500Hz）が無圧縮で約120 Kbytesになり、短時間（15～30秒）で伝送された。また、ベッドサイド・モニタによる連続波形が、ほぼ遅延なくリアルタイムで伝送可能であった。画像については、ネットワーク・カメラに内蔵されたハードウエア・エンコーダ

により、動画（MPEG4またはMotion JPEG、64〜192 Kbps）および静止画（JPEG）の配信が可能であった。カメラの向きやズーム等は、救急車側ではなく、病院側からリモート・コントロールされた。

命を救う病院前救護

プロトタイプの開発、研究室でのテスト、および救急車への試験的搭載による実証実験により、既存の医療機器および情報通信機器の組み合わせによるモバイルテレメディシンの実現可能性が確認された。

AMIの初期治療をCCUから地域の病院前救護に拡大する考え方は、66年に北アイルランドのBelfastで確立され、米国では医師常駐型からパラメディック育成型として消防、警察、民営救急隊等にも拡がりをみせている。院外心停止対策ではシアトル州キング郡のMedicOneが有名である。

除細動とCPR（心肺蘇生法）は命を救う。我々は、吹田市を皮切りに、病院前救護の支援と適切な搬送による早期再灌流療法の実施を全国に普及させるべく、"Heart & Brain Watch"構想を提唱している。

○結　語

循環器救急における現場のニーズを検討し、適切な搬送および病院前救護の改善を目的としたモバイル・テレメディシン開発の必要性を明らかにした。

技術的可能性として、循環器救急医療に必要な情報の符号化、データの圧縮・伸張技術、および利用可能な移動体通信の現状と将来性について検討し、救急車を救急指令台や病院とリアルタイムに結び、救急救命士の活動を支援するプロトタイプ作成と研究室での動作確認、救急車での実証実験を行った。

今後7つのS（Standard, Simple, Speed, Scalability, Security, Safety, Study）や費用対効果に配慮しつつ、ベンチ・テスト、フィールド・テストおよびアウトカム・リサーチの実施を予定している。

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NTTコムウエア：東京都港区
松下電器産業：大阪府門真市
日本光電工業：東京都新宿区
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医師主導型治験を支える医療機関のサポート体制

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はじめに

平成15年度は，改正薬事法施行および臨床研究倫理指針告示という臨床試験の実施体制に関する大きな変革の年であった。臨床試験研究は医療の質向上のために必要不可欠であるが，選択肢の1つとして医師主導の治験が新たに加わった。その実施は容易ではなさそうであるが，医療機関では被験者保護を念頭に，スピード・質・コストを改善するための簡素で標準的なサポート体制をつくることが重要である。

背　景

ゲノム時代を迎え，診断や治療のパラダイム・シフトが始まった[1)-6)]。バイアスや利益相反は永遠の課題であり，基礎実験や探索的研究の結果から導かれた仮説が検証的試験により，ときには覆る[7)-9)]ことから，患者にとっての真の利益を評価するために，臨床試験研究の重要性はますます高まっている[10)-12)]。

わが国でも，バイオテクノロジー戦略会議の最終答申[13)]や，文部科学省，厚生労働省の全国治験活性化3カ年計画[14)]などで，諸外国との比較で問題視されている臨床研究のスピード・質・コストを改善するための基盤整備がうたわれている。

平成15年度には改正薬事法施行および臨床研究指針告示という大きな変化があった。世界的な被験者保護の流れと合わせ，医療機関における臨床試験のサポート体制の強化が急務となっている。

臨床研究と治験および医師主導型治験

「治験」とは薬事法第2条により定義されている言葉で，厚生労働大臣に治験届を提出した上で，新しい医薬品・医療機器の承認を得るため，科学的見地からの審査に必要な実証データの収集を目的として，ヒトを対象に実施される臨床試験のことである（図1）。

わが国においては，治験にまつわる金銭面の疑惑やデータの信頼性などが問題とされ，主として保険診療に従事する医療機関にとってのインセンティブや，基礎研究と比較して学術論文などの成果につながりにくいなどの理由から，医師の治験への関心は高いとは言えなかった。

しかしながら，エビデンスに基づく医療（EBM）という考え方が普及するにつれ，患者，医療従事者の双方で臨床疫学的な情報の共有が進みつつある。疾患の頻度，自然歴，現在ある最善の治療についての検討がなされれば，必然的に諸外国で標準的とされる医薬品・医療機器が未承認あるいは