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Case Report

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Successful cinacalcet treatment of refractory secondary hyperparathyroidism due to multiple lung parathyroid adenomas

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Abstract

We describe a 56-year-old woman who presented with end-stage renal disease due to pregnancy-induced hypertension and secondary hyperparathyroidism (sHPT). She had started hemodialysis and underwent a subtotal parathyroidectomy (PTx). However, intact parathyroid hormone (iPTH) levels increased gradually. Eventually, she underwent a second PTx. However, therapy failed to significantly decrease iPTH levels. A third PTx was performed, but no pathological parathyroid tissue was found. Computed tomography scan indicated the presence of multiple ectopic lung nodules and 26 nodules were surgically removed from the left lung. Despite surgical treatment, iPTH levels remained high. Additional maxacalcitol failed to decrease iPTH levels, cinacalcet was then started. iPTH levels decreased and the cinacalcet dose could be reduced to maintenance doses of 60 mg/day. Throughout the 1.6 years of treatment, serum iPTH, alkaline phosphatase (ALP) and bone alkaline phosphatase (BAP) were normalized. As a consequence, bone pain gradually disappeared. Bone mineral density (BMD) was improved by administration of cinacalcet. In conclusion, cinacalcet was effective in this patient with refractory and inoperable sHPT. In addition, it improves their BMD and relieves bone pain.

Keywords: cinacalcet; haemodialysis; hyperparathyroidism; ectopic parathyroid adenoma

Introduction

Progressive and refractory secondary hyperparathyroidism (sHPT) is a serious complication of end-stage renal disease (ESRD) accompanied by cellular transformation of the parathyroids, which often results in aggressive growth of the glands and decreased expression of both vitamin D and calcium-sensing receptors (CaSR) on their surfaces [1]. Vitamin D and phosphate binders are widely used to prevent the progression of sHPT. However, these conventional treatments often fail to control severe sHPT. In such cases, parathyroidectomy (PTx) is required. However, the frequent growth of sHPT tumours occurring outside the neck, e.g. in the lungs, is not always treated surgically [2]. Herein, we describe successful treatment with cinacalcet of refractory sHPT due to multiple lung parathyroid adenomas.

Case report

A 56-year-old woman was referred to our hospital in October 2003 with a diagnosis of ESRD due to pregnancy-induced hypertension and sHPT. According to her medical records, she had started haemodialysis in March 1994 and underwent a subtotal PTx in the same year. During surgery, a parathyroid adenoma involving the right lower and left lower parathyroid glands was removed, and PTx was successful. A gradual increase in intact parathyroid hormone (iPTH) levels was, however, observed 6 years later. Eventually, in February 2001, she underwent a second PTx. The two evidently enlarged parathyroid glands (each 5 × 10 mm in size) were removed. Nodules were well circumscribed, solid and yellow–white in colour. Histological examination showed parathyroid adenomas with oxyphilic cytoplasm but neither necrosis nor vascular invasion. There was no histopathological evidence of malignancy. However, therapy failed to significantly decrease iPTH levels, which ranged from 1500–2000 pg/mL. A third PTx was performed, but no pathological parathyroid tissue was found.

In July 2002, a computed tomography (CT) scan indicated the presence of multiple ectopic lung nodules. In September 2002, 26 nodules were surgically removed from the left lung. Intraoperative histological examination showed parathyroid adenoma. Despite surgical treatment, iPTH levels remained high. In addition, maxacalcitol was administered intravenously at a dose of 20 μg after each dialysis session. Nevertheless, iPTH levels kept increasing, and were in the 2000–4000 pg/mL range. She mainly complained of worsening bone pain and loss of height (from 162–154 cm). In October 2003, we identified the right mediastinal parathyroid glands by MIBI (99mTc-sesta-MIBI) scintigraphy. In November 2003, CT scan detected the presence of multiple ectopic nodules mainly in the right lung (Figure 1). She declined further surgical treatment due to its

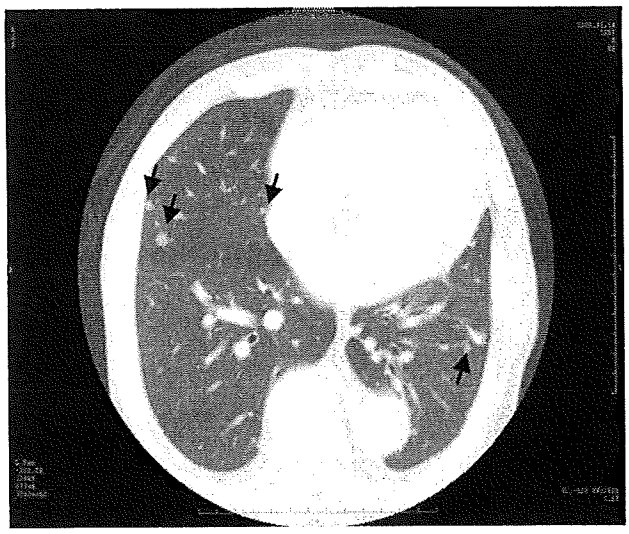

Fig. 1. Chest CT scan revealed multiple lung nodules indicative of multiple lung adenomas.

probable futility; we thus decided to administer cinacalcet as oral therapy prior to its market launch in Japan. However, 1 year was required for acquisition of this drug. For this reason, her height decreased another 10 cm during the 1-year-waiting period.

Cinacalcet was started at a daily dose of 30 mg in September 2004, and titrated up to a dose of 120 mg/day. The patient remained on this high dose for 58 weeks; subsequently, her iPTH levels decreased, and the dose could be reduced stepwise to maintenance levels of 60 mg/day. During treatment with cinacalcet, the patient also regularly received other medications, including a phosphate binder (sevelamer, 5250–6750 mg/day), precipitated calcium carbonate (500–1500 mg/day) and maxacalcitol (22-oxacalcitriol 60 μg/week). Unfortunately, this calcium carbonate dose could not be increased due to the patient's refusal.

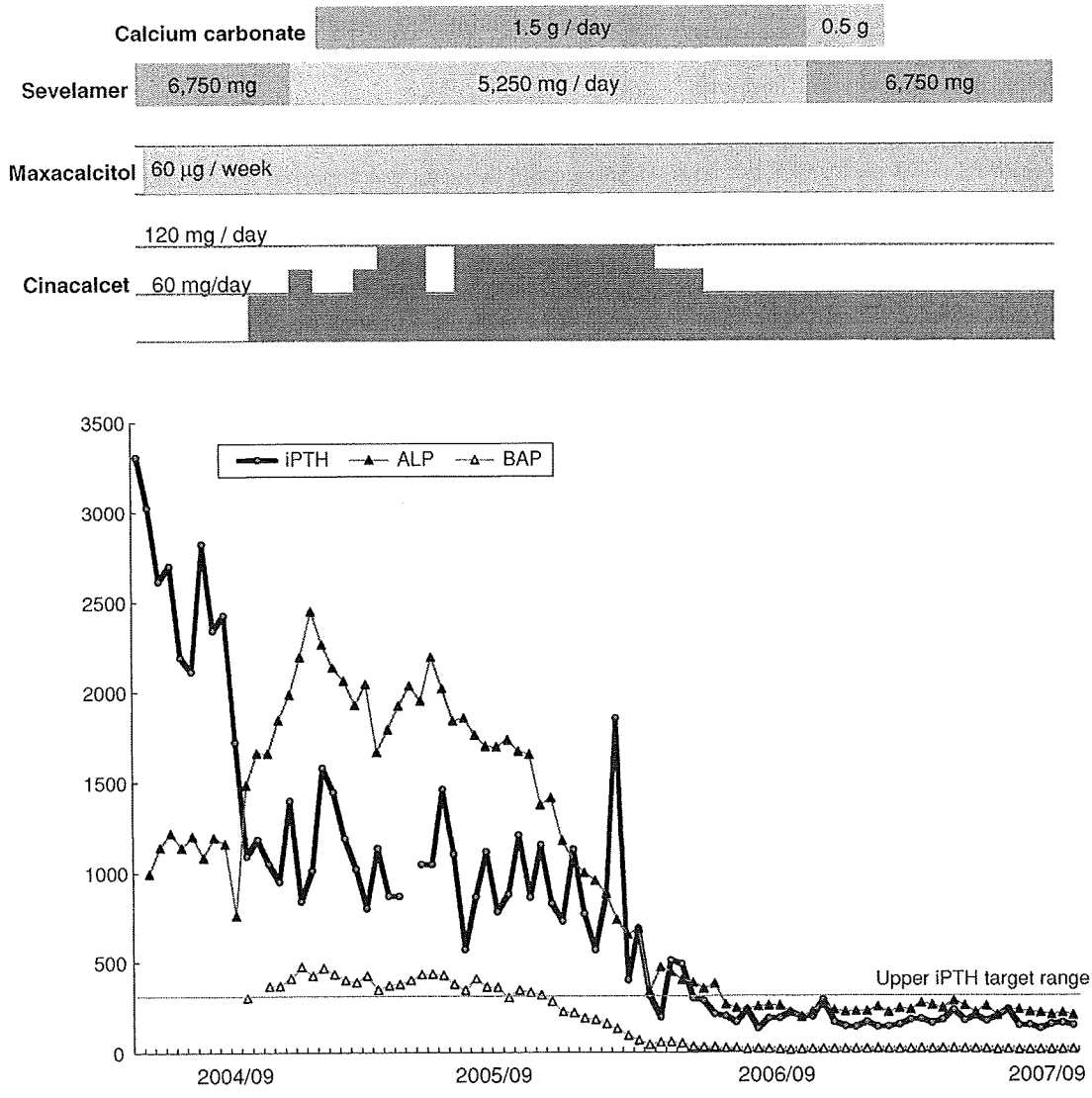

Fig. 2. Changes in Ca, P, ALP, BAP and iPTH, before versus after treatment with cinacalcet. Measured serum Ca levels were adjusted by albumin levels as follows: when they were <4.0 g/dL: Ca = measured calcium levels + (4.0 − albumin levels) mg/dL.

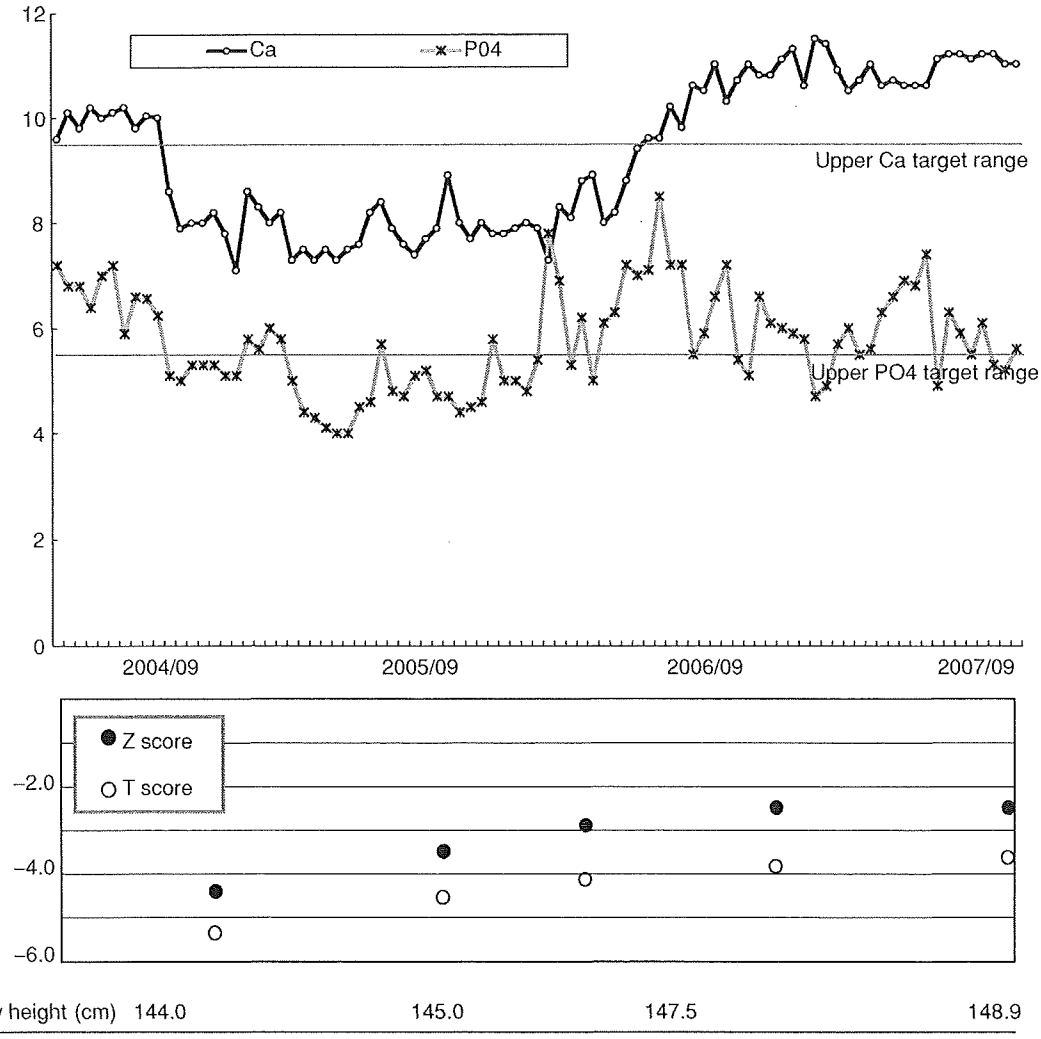

Fig. 3. Changes in Ca, P, and BMD, before versus after treatment with cinacalcet.

Throughout the 1.6 years of treatment, serum levels of iPTH, alkaline phosphatase (ALP) and bone alkaline phosphatase (BAP) were persistently decreased (Figure 2), while Ca and P steadily increased (Figure 3). Thereafter, with the rise of Ca (>8 mg/dL), iPTH (which stayed in the 750–1500 pg/mL range) ultimately fell to 150–200 pg/mL. Similarly, ALP and BAP were normalized. As a consequence, bone pain gradually disappeared. Bone mineral density (BMD) was improved by administration of cinacalcet. Before this treatment, the BMD of the distal one-third of the radial bone on dual-energy x-ray absorptiometry (DEXA) was 0.349 g/cm^2 (T score, −5.7 SD), but after 3 years of cinacalcet administration, had improved to 0.432 g/cm^2 (T score, −3.7 SD).

Discussion

We have demonstrated cinacalcet therapy to be effective for refractory sHPT due to multiple lung parathyroid adenomas. Our case showed severe hypocalcaemia and hypophosphataemia with increases in ALP and BAP after initiation of cinacalcet, similar to the so-called hungry bone syndrome occasionally seen in classic SHPT following PTx [3]. In the hungry bone syndrome, the equilibrium between calcium efflux from and influx into the bone matrix is severely disrupted accompanied by rapidly falling PTH. Increased osteoclastic activity associated with osteitis fibrosa, elevated ALP and large parathyroid gland volume at the time of resection are predictors of developing this syndrome [3,4]. Consequently, it has been suggested that calcimimetics may induce this phenomenon [5,6].

Cinacalcet is an allosteric modulator of the CaSR. Cinacalcet increases CaSR sensitivity, lowers the threshold for activation by calcium and decreases PTH secretion [7]. A rat model and a clinical study demonstrated that the inverse sigmoid curve between plasma calcium ion (Ca2+) and serum PTH levels was simply shifted leftward by calcimimetic infusion [7,8]. This result suggested that lowering plasma Ca2+ might induce a conformational change of the CaR, resulting in dissociation from G proteins

or loss of the binding site for cinacalcet. Our present case had prolonged hypocalcaemia. During severe hypocalcaemia, iPTH levels remained in the range between 750 and 1500 pg/mL. Thereafter, coinciding with the rise of Ca (>8 mg/dL), iPTH levels ultimately fell to 150–200 pg/mL. Her clinical course appeared to be consistent with this hypothesis.

In conclusion, cinacalcet was effective in this patient with refractory and inoperable sHPT. In addition, it improves their BMD and relieves bone pain.

Conflict of interest statement. None declared.

References

1. Tominaga Y. Management of renal hyperparathyroidism. *Biomed Pharmacother* 2000; 54(Suppl 1): 25–31
2. Tseng CC, Huang JJ, Wang MC *et al.* Parathyroid carcinoma with multiple lung metastases. *Nephrol Dial Transplant* 1999; 14: 449–451
3. Cruz DN, Perazella MA. Biochemical aberrations in a dialysis patient following parathyroidectomy. *Am J Kidney Dis* 1997; 29: 759–762
4. Lazar ES, Stankus N. Cinacalcet-induced hungry bone syndrome. *Semin Dial* 2007; 20: 83–85
5. Goodman WG, Hladik GA, Turner SA *et al.* The calcimimetic agent AMG 073 lowers plasma parathyroid hormone levels in hemodialysis patients with secondary hyperparathyroidism. *J Am Soc Nephrol* 2002; 13: 1017–1024
6. Torres PU. Clinical experience with cinacalcet HCl. *Nephrol Dial Transplant* 2004; 19: v27–v33
7. Nagano N. Pharmacological and clinical properties of calcimimetics: calcium receptor activators that afford an innovative approach to controlling hyperparathyroidism. *Pharmacol Ther* 2006; 109: 339–365
8. de Francisco AL, Izquierdo M, Cunningham J *et al.* Calcium-mediated parathyroid hormone release changes in patients treated with the calcimimetic agent cinacalcet. *Nephrol Dial Transplant* 2008; 23: 2895–2901

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Prevalence and Cardiovascular Features of Japanese Hemodialysis Patients with Fabry Disease

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Key Words
Fabry disease · Japanese hemodialysis patients · Cardiovascular abnormalities

Abstract
Background: Fabry disease (FD) is a rare disease and one of the causes of progressive renal dysfunction. It results from an X-linked deficiency of α-galactosidase A activity. It has been reported that its prevalence is much higher in hemodialysis patients than in the general population. However, its prevalence in Japanese hemodialysis patients and cardiovascular manifestations remain unclear. ***Methods:*** We screened the α-galactosidase A activity of 1,024 Japanese hemodialysis patients using a dried blood spot test. Patients with a low α-galactosidase A activity were assessed clinically, and a genetic study of the α-galactosidase A gene was performed for these patients. Furthermore, patients with FD underwent detailed cardiovascular examination. ***Results:*** Forty-six patients had low α-galactosidase A activity, and 1 man and 2 women had α-galactosidase A mutations (0.29%). All of these patients had a previously identified mutation (E66Q). The result of detailed cardiovascular examination showed that 2 patients had significantly impaired coronary flow reserve, reduced myocardial contraction and relaxation tissue Doppler velocities, and left ventricular hypertrophy. ***Conclusions:*** Measurement of the α-galactosidase A activity and the results of a genetic analysis indicated that the prevalence of FD in our hemodialysis patients was 0.29% (0.16% in men and 0.5% in women). Furthermore, comprehensive examination detected cardiovascular abnormalities in Japanese hemodialysis patients with FD.

Introduction

Recently, accumulating evidence has indicated a close relationship between pathologies of the kidney and heart, and both organs should be examined when there is a problem, for example in the case of mild renal dysfunction and/or the presence of albuminuria, which are powerful cardiovascular disease risk factors [1, 2].

Fabry disease (FD) is known to affect both the kidney and heart. FD is an X-linked recessive lysosomal storage disorder caused by a defect in the gene encoding lyso-

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somal α-galactosidase A. Patients with classic FD manifest a variety of clinical symptoms, such as acroparesthesias, angiokeratoma, hypohidrosis, corneal opacities, stroke, renal disorder, and cardiac abnormalities. Patients with these symptoms have an extremely high mortality rate. It has been reported that the prevalence of FD is 1 in 40,000–117,000 males [3, 4]. However, several studies have reported that the prevalence may be much higher in dialysis patients, as high as 1.2% [5–11]. In addition, it seems that the prevalence of females with FD is also much higher among hemodialysis patients.

We speculated that FD may be more prevalent in the hemodialysis patient population than in the general population in Japan as well as in the United States and Europe. Therefore, in the present study, we investigated the prevalence and features, such as cardiovascular abnormalities, of Japanese hemodialysis patients with FD.

Materials and Methods

Study Population and Design

From December 2006 to December 2007, a total of 1,024 patients with end-stage renal disease (ESRD; chronic kidney disease, CKD, stage 5D) from 17 different clinical facilities in Hyogo Prefecture in Japan participated in this study. All patients were receiving hemodialysis treatment two or three times a week. Patients with FD and those who were undergoing peritoneal dialysis were excluded from the present study. Furthermore, patients who did not agree to undergo measurement of α-galactosidase A activity and a genetic analysis and those who did not receive a remeasurement of α-galactosidase A activity were also excluded. The protocol was approved by the Institutional Review Committee for the Protection of Human Subjects in Research at Kobe University, and all participants provided informed consent according to the Declaration of Helsinki.

We measured the α-galactosidase A activity of 1,024 hemodialysis patients using a dried blood spot test. α-Galactosidase A activity <20 Agal U was identified as abnormally low. In a separate study using this cutoff value, we found 26 mutations in 30 subjects with low α-galactosidase A activities in a screening test of about 120,000 newborns [Nakamura et al., in preparation]. Based on the study, the estimated specificity and sensitivity of the test was nearly 100%. Patients with α-galactosidase A activity <20 Agal U had blood drawn again for the remeasurement of the α-galactosidase A activity on another day. Patients with a low α-galactosidase A activity after remeasurement were assessed clinically, and they underwent a genetic study of the α-galactosidase A gene. Furthermore, patients with FD underwent a comprehensive cardiovascular examination that included carotid ultrasonography, measurement of pulse wave velocity, Holter electrocardiogram, magnetic resonance imaging (MRI) of the brain, Doppler imaging of myocardial tissue, exercise-stress myocardial scintigraphy, and measurement of coronary flow reserve (CFR). However, one patient (case 3) refused some of these procedures.

Measurement of α-Galactosidase A Activity

Venous blood was collected from patients before dialysis. Four drops of the blood were transferred to a filter paper, allowed to dry at room temperature, and stored at 2–4°C until they were sent to Kumamoto University in Kumamoto, Japan. The α-Galactosidase A activity was determined from the dried blood spots with a fluorescence assay using 4-methylumbellifenyl.

Genetic Study of α-Galactosidase A Gene

Genomic DNA was extracted from peripheral blood leucocytes. DNA regions of the α-galactosidase A gene were analyzed by polymerase chain reaction after amplifying each of the seven α-galactosidase A exons and sequencing the opposite strand.

Echocardiographic Studies

Two-dimensional guided M-mode echocardiography was conducted to measure echocardiographic parameters and the left ventricular mass index. Tissue Doppler imaging was conducted in the pulse Doppler mode to record mitral annulus velocities at the septal and lateral corners. Systolic (Sa), early diastolic (Ea), and late diastolic (Aa) tissue Doppler velocities were measured, and the Ea/Aa ratio and the dimensionless parameter E/Ea were computed at both corners of the mitral annulus. For each measurement, five beats were averaged.

CFR Measurement

CFR was measured using transthoracic Doppler echocardiography as described previously [12]. Analysis of the coronary flow velocity was conducted off-line by tracing the contour of the spectral Doppler signal using an ultrasound system computer. Mean diastolic velocity (MDV) was measured at baseline and peak hyperemic conditions. Measurements were averaged over three cardiac cycles. CFR was defined as the ratio of hyperemic to basal MDV. We adopted a CFR <2.0 as the cutoff value for the presence of significant coronary microvascular disease as in previous studies [12].

Statistical Analysis

Values are presented as mean ± SD.

Results

Study Patients

We screened 625 men and 399 women in this study. Sixty-one percent of the study patients were men. The average age of the patients was 62 ± 12 years; the youngest patient was 19 years and the oldest patient was 91 years old.

α-Galactosidase A Activity and Genetic Analysis in Study Patients

The average α-galactosidase A activity of the study patients was 30.3 ± 11.6 Agal U. The first measurement indicated that 117 patients had an α-galactosidase A activity <20 Agal U; 907 patients had activity >20 Agal U.

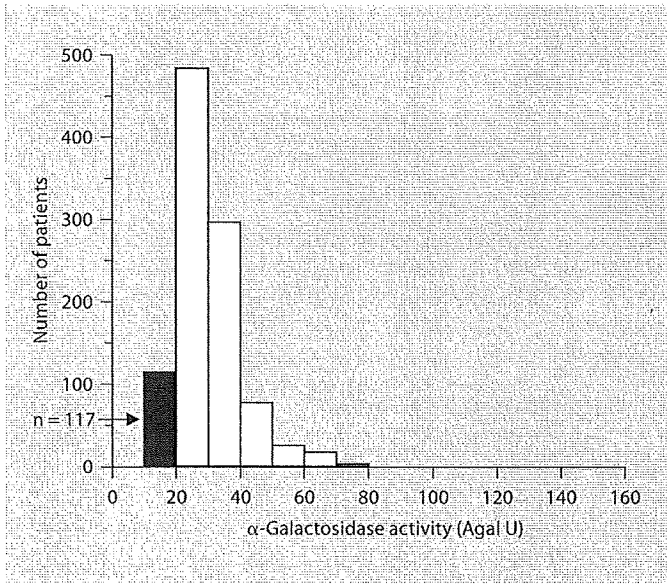

Fig. 1. Distribution of α-galactosidase A activity in all the patients in the study. The mean ± SD α-galactosidase A activity was 30.3 ± 11.6 Agal U.

Table 1. Patient characteristics

	Case 1	Case 2	Case 3
Age, years	45	51	83
Sex	female	male	female
BMI	26.4	21.2	19.3
HD duration, years	12	23	4
Cause of ESRD	unknown	unknown	unknown
Hypertension	+	–	–
DM	+	–	+
Hyperlipidemia	–	+	–
CAD	–	–	–
CHF	–	–	–
Stroke	+	+	–
Acroparesthesia	–	–	–
Hypohidrosis	–	–	–
Angiokeratoma	–	–	–
Corneal opacities	–	–	–
Chest pain	+	–	–

BMI = Body mass index; HD = hemodialysis; DM = diabetes mellitus; CAD = coronary artery disease; CHF = congestive heart failure.

The distribution of α-galactosidase A activity in all of the study patients is shown in figure 1. After remeasurement in the 117 patients with a low α-galactosidase A activity, 46 patients (25 men and 21 women) still had a low α-galactosidase A activity. We performed a genetic analysis in these 46 patients. The results showed that 1 man and 2 women had a missense mutation (fig. 2). All 3 of these patients had the same mutation (E66Q), which had been reported previously [13, 14].

Assessment of Clinical Characteristics and Biochemical Markers

The characteristics of the patients are shown in table 1. None of the patients had classic symptoms of FD. In addition, the cause of ESRD was unknown in all of the patients, and none of the patients had a past history of either coronary artery disease or congestive heart failure. One patient had angina-like chest pain (case 1). We also researched the family pedigrees of the patients (fig. 3). Unfortunately, some of the families refused to undergo screening and a genetic analysis. However, 4 patients had newly diagnosed FD, and we found that all of them had a low α-galactosidase A activity (average activities; 14.3 ± 2.2 Agal U). The mother of patient 1 had a history of cerebral hemorrhage. In addition, the mother of patient 2 had maintenance hemodialysis therapy and a history of cerebral infarction. However, since other patients did not have manifest renal and cardiac symptoms, we did not perform renal or cardiac biopsy for these patients.

Biochemical data are shown in table 2. For comparison with α-galactosidase A activity measured using the dried blood spot test, we also measured α-galactosidase A activity in leukocytes. The results showed that one patient (case 2) had a low α-galactosidase A activity in leukocytes (32.1 nmol/mg protein/h). Plasma GL-3 levels were normal in all of the patients, even in those with low α-galactosidase A activity. Average α-galactosidase activities in male and female patients were 30.3 ± 10.4 and 30.4 ± 13.6 Agal U, respectively, and there was no significant difference.

Evaluation of Cardiovascular Abnormalities in Patients with FD

The electrocardiogram showed an ST depression, a negative T wave, and a prolonged QT interval in one patient (case 1), but 2 other patients had no significant abnormalities (table 3). The left ventricular mass index increased and systolic function decreased in 2 patients (cases 1 and 2), and the Ea/Aa ratio decreased in these patients (table 4). Unfortunately, we were able to perform tissue Doppler echocardiography and measure CFR in only 2 patients (cases 1 and 2). Tissue Doppler imaging

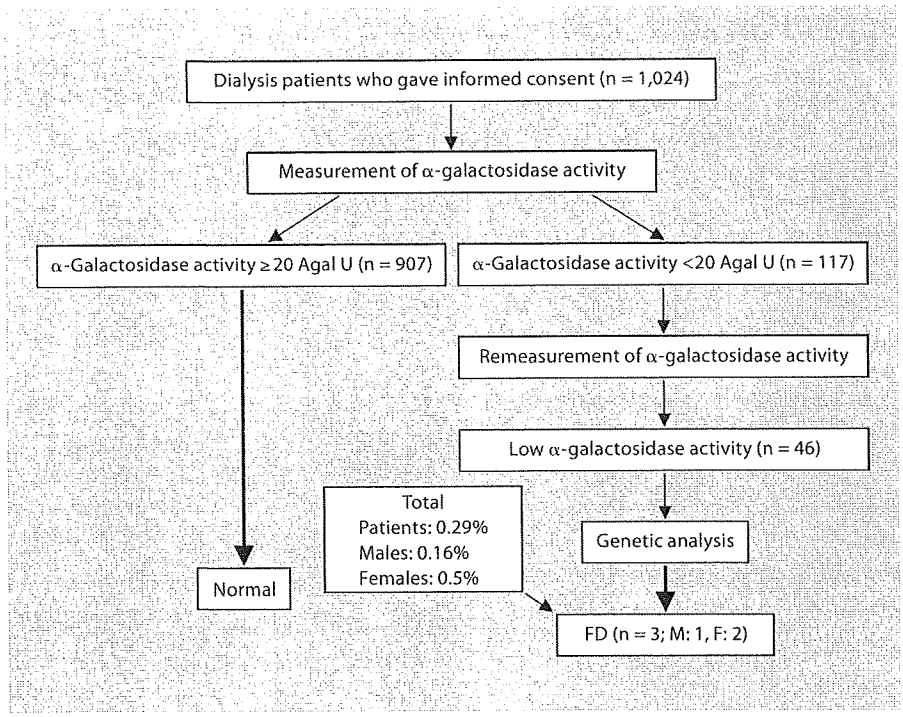

Fig. 2. Flow chart for screening dialysis patients with FD.

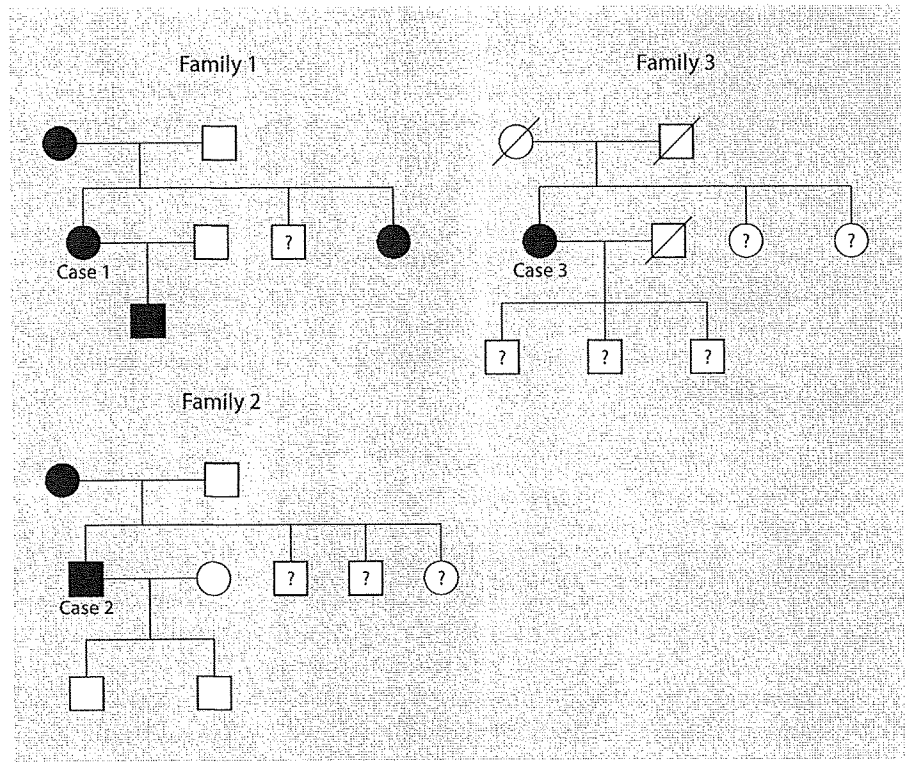

Fig. 3. Family pedigrees of the patients with FD in this study. Males are denoted by squares, females by circles. Filled symbols indicate those with FD. Open symbols indicate normal patients. Symbols with a question mark indicate patients with unknown disease status. The dashes indicate deceased patients.

Table 2. Biochemical data

	Case 1	Case 2	Case 3	Normal values
TP, g/dl	6.8	7.9	6.4	6.4–8.2
Alb, g/dl	3.9	3.6	3.3	3.5–5.2
T-chol, mg/dl	125	158	131	130–240
TG, mg/dl	178	112	140	30–180
HDL, mg/dl	38	37	30	≥40
HbA_{1c}, %	6.1	4.6	7.1	4.3–5.8
Hb, g/dl	11.4	12.1	10.3	
Ca, mg/dl	8.9	10.4	8.5	
P, mg/dl	5.2	6.7	3.7	
Ca × P, mg^2/dl^2	46.3	69.7	31.5	
int-PTH, pg/ml	210	56	112	
α-Galactosidase activity				
Dried blood spot, Agal U	16.9	15.2	12.9	≥20.0
Leukocyte, nmol/mg protein/h	61.2	32.1	NE	49.8–116.4
GL-3, μg/ml	4.3	<0.3	4.1	≤7.0
BNP, pg/ml	321.5	272.2	503.1	≤18.4
hsCRP, mg/dl	0.30	0.23	0.12	<0.30
Homocysteine, nmol/ml	36.9	40.2	NE	3.7–13.5
ET-1, pg/ml	3.20	2.13	NE	≤2.30

TP = Total protein; Alb = albumin; T-chol = total cholesterol; TG = triglyceride; HDL = high-density lipoprotein cholesterol; HbA_{1c} = hemoglobin A_{1c}; Hb = hemoglobin; int-PTH = intact parathyroid hormone; GL-3 = globotriaosylceramide; BNP = brain natriuretic peptide; hsCRP = high-sensitivity C-reactive protein; ET-1 = endothelin-1.

Table 3. Electrocardiogram findings

	Case 1	Case 2	Case 3
sV1 + rV5, mm	48	30	22
ST depression	+	–	–
PR, ms	148	172	142
QRS, ms	86	94	76
QTc, ms	445	427	426
Abnormal Q wave	–	–	–
LBBB	–	–	–
RBBB	–	–	–
AF	–	–	–
VT	–	–	–
VPC	Lown 1	Lown 1	Lown 1

LBBB = Left bind brunch block; RBBB = right bind brunch block; AF = atrial fibrillation; VT = ventricular tachycardia; VPC = ventricular premature conduction.

Table 4. Echocardiographic parameters

	Case 1	Case 2	Case 3
LVDd, mm	49	47	41
LVDs, mm	37	33	25
IVST, mm	16	12	8
LVPWT, mm	13	12	12
FS, %	24	29	39
EF, %	58	57	68
LVMI, g/m^2	203.2	151.9	111.7
E/A	0.77	0.65	0.41
DcT, ms	346	212	235
IVCD, mm	16	13	12

LVDd = Left ventricular diastolic diameter; LVDs = left ventricular systolic diameter; IVST = intraventricular septum thickness; LVPWT = left ventricular posterior wall thickness; FS = fractional shortening; EF = ejection fraction; LVMI = left ventricular mass index for body surface area; DcT = deceleration time; IVCD = inferior vena cava diameter.

Table 5. Tissue Doppler imaging data

	Case 1	Case 2	Case 3
LV lateral wall			
Sa, cm/s	4.05	7.05	NE
Ea, cm/s	1.67	5.96	NE
Aa, cm/s	1.68	3.55	NE
Ea/Aa, cm/s	0.99	6.42	NE
E/Ea, cm/s	37.1	6.71	NE
Intraventricular septum			
Sa, cm/s	2.28	5.55	NE
Ea, cm/s	1.64	6.29	NE
Aa, cm/s	3.90	7.78	NE
Ea/Aa, cm/s	0.42	0.81	NE
E/Ea, cm/s	37.8	6.36	NE
Strain rate	1.59	2.11	NE

LV = Left ventricle; NE = not examined.

Table 6. Coronary flow reserve

	Case 1	Case 2	Case 3
Baseline SBP, mm Hg	212	135	NE
Baseline DBP, mm Hg	88	73	NE
Hyperemia SBP, mm Hg	147	99	NE
Hyperemia DBP, mm Hg	63	46	NE
Baseline HR, bpm	73	76	NE
Hyperemia HR, bpm	92	76	NE
Baseline MDV, cm/s	47.4	29.8	NE
Hyperemia MDV, cm/s	83.9	43.2	NE
Coronary flow reserve	1.77	1.45	NE

SBP = Systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate.

Table 7. Results of other cardiovascular examinations

	Case 1	Case 2	Case 3
Pulse wave velocity			
baPWV, m/s	2,792	1,914	NE
ABI	1.15	1.36	NE
TBI	0.64	0.85	NE
Carotid ultrasonography			
Max. IMT, mm	1.8	0.7	1.2
Plaque score	4.3	9.5	13.4
CA stenosis	–	–	+ R-ICA 59% L-ICA 60%
Brain MRI			
White matter lesion	+	+	NE

baPWV = Brachial-ankle pulse wave velocity; ABI = ankle-brachial index; TBI = coronary flow reserve; IMT = intima-media thickness; CA = carotid artery; R-ICA = right internal carotid artery; L-ICA = left internal carotid artery.

showed decreased myocardial velocities and strain rates (table 5). The results of the CFR measurements are shown in table 6. We performed exercise stress myocardial scintigraphy in these 2 patients, but ischemia was not observed. Despite the absence of ischemia, these patients had a decreased CFR. Interestingly, in addition to the impairment in CFR, one of the patients (case 1) had both angina-like chest pain and elevated plasma endothelin-1 levels, which might have played an active role in the disease process of microvascular angina. Carotid ultrasonic examination showed moderate to severe atherosclerosis of the carotid artery in all of the study patients (table 7). Two patients had a history of stroke (cases 1 and 2). Therefore, we performed MRI on these 2 patients. In both patients, MRI of the brain indicated an old cerebral infarction and a white matter lesion, which are typical findings of FD (fig. 4).

Discussion

Our study showed the following findings: (1) the prevalence of FD in dialysis patients was 0.29% (0.16% in men and 0.5% in women); (2) all of the patients had a previously identified mutation (E66Q); and (3) comprehensive cardiovascular examinations identified some cardiovascular abnormalities, although none of the patients had classic symptoms other than renal involvement.

Several studies have reported the prevalence of FD in dialysis patients. It was reported by Kotanko et al. [8] to be 0.16% (n = 4) among 2,480 Austrian dialysis patients and by Merta et al. [10] to be 0.15% (n = 5) among 3,370 Czech Republic hemodialysis patients. On the other hand, some small studies from Japan suggested a prevalence of 0.16–1.2% among hemodialysis patients [5, 6, 9]. However, few of them included female dialysis patients. Thus, the prevalence may be much higher in dialysis patients than in the general population, probably because hemodialysis patients with FD may lack typical manifestations of the disease. Some variant types of FD have been identified, the manifestations of which are primarily limited to the heart, kidney, and brain [9, 15–19]. Therefore,

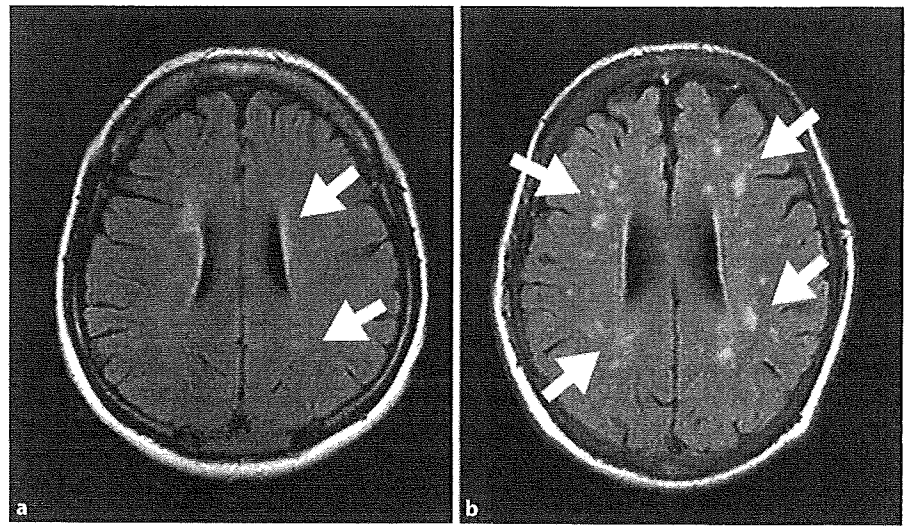

Fig. 4. Brain MRI of cases 1 (**a**) and 2 (**b**). Arrows point to white matter lesion.

it is very difficult to identify hemodialysis patients with FD because the variant types of FD lack the classic symptoms of the disease. In the present study, we performed a screening test in >1,000 Japanese hemodialysis patients and identified 3 patients with FD. Furthermore, to investigate a true prevalence of FD in Japanese dialysis patients, we thought that a screening test needed to be performed that would include female ESRD patients.

Screening tests are available for identifying patients with FD. Most previous screening studies used a plasma α-galactosidase assay, and others measured α-galactosidase A activity in leukocytes. Currently, about 300,000 patients are receiving maintenance hemodialysis in Japan. Performing screening tests in all of these patients would be very costly. Furthermore, because most of these patients receive dialysis therapy in small clinics, it would be very difficult to perform complicated screening tests. Therefore, in the present study, we performed screening tests for FD using an α-galactosidase blood spot test, because it is simple and inexpensive. Andrade et al. [20] performed a screening test using a plasma enzyme assay, which is the most commonly used screening test. However, they did not diagnose any new cases of FD in 499 CKD patients probably because of false-negative results and intraindividual variations. On the basis of such limitations, we performed a screening test involving repeated measurement of α-galactosidase activity combined with a genetic analysis and identified 3 patients with FD. Accordingly, as Andrade et al. [20] noted, we should pay attention to factors such as sample type, laboratory method design, and data analysis. We believe that the α-galactosidase blood spot test is a suitable screening method for FD.

Two of our patients with FD had mild systolic dysfunction, diastolic dysfunction, and left ventricular hypertrophy (cases 1 and 2). Interestingly, we found that these patients had some reported factors associated with FD. Elliott et al. [21] reported that patients with FD have impaired CFR, possibly because of coronary microvascular dysfunction. Certainly, we have to take into account the influence of ESRD. Some studies have shown that ESRD patients also have decreased CFR, despite having no significant coronary artery stenosis [22, 23]. Although it has been reported that CFR of dialysis patients is 1.9–2.0 [22, 23], it was lower in our patients. Therefore, we speculated that the cause of decreased CFR in our patients was endothelial dysfunction due to FD. Several studies have shown that conventional measurements of global cardiac function, such as ejection fraction and fractional shortening, are not sensitive enough to detect impaired cardiac function in patients with variant types FD [24, 25]. Thus, recent studies have used tissue Doppler myocardial imaging because this method enables the detection of reduced myocardial function before manifesting cardiac dysfunction in FD [24, 25]. These studies showed that myocardial velocities and strain rate were dramatically reduced in patients with FD (Sa <10 cm/s, Ea <10 cm/s). The results of the present study agree with these reports. Rolfs et al. [15] reported that 4% of patients with cryptogenic stroke had FD. In our study, 2 of our patients also had a history of cryptogenic stroke. In addition, it has been reported that

patients with FD have cerebral white matter lesions, and MRI of the brain can detect these lesions even at an early age [26]. Our 2 patients with a history of stroke had an old cerebral infarction and severe white matter lesions on MRI.

It was difficult to determine whether the cause of renal and cardiovascular abnormalities in our patients was FD. As mentioned above, the cause of ESRD in our patients was unknown, and these patients did not have typical symptoms of classic FD, although 2 of them had a history of cerebral infarction. The cardiac abnormalities in our patients were relatively mild. On the basis of a case-note review and the results of our investigation, we suspected that the renal and cardiac dysfunction in our patients was due to FD. Therefore, we consider these patients to have a variant type of FD. Moreover, all of our study patients with FD had the same genetic mutation (E66Q), which results in the substitution of glutamine for glutamic acid at residue 66. Patients with this mutation are frequently reported in Japan [13]. Ishii et al. [14] first reported that this mutation causes a classic FD if it coexists with another mutation R112C. Our patients, as well as patients in previous studies, had only one mutation, E66Q. As a consequence, they had low α-galactosidase A activity and did not have severe symptoms.

Conclusion

It is important that patients with FD be identified so that the family members of these patients can be screened for the disease. Furthermore, comprehensive examinations are able to detect cardiovascular abnormalities in hemodialysis patients with FD. Recently, patients with FD have been started on enzyme replacement therapy (ERT) in many countries. Various studies have shown that ERT can delay the impairment of cardiac and renal function [27, 28]. Therefore, it is important to identify patients with FD and the complications of this disease as early as possible so that ERT can be initiated in these patients.

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References

1 Ritz E, McClellan WM: Overview: increased cardiovascular risk in patients with minor renal dysfunction: an emerging issue with far-reaching consequences. J Am Soc Nephrol 2004;15:513–516.

2 Gerstein HC, Mann JF, Yi Q, Zinman B, Dinneen SF, Hoogwerf B, Halle JP, Young J, Rashkow A, Joyce C, Nawaz S, Yusuf S, HOPE Study Investigators: Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals. JAMA 2001;286:421–426.

3 Desnick RJ, Brady R, Barranger J, Collins AJ, Germain DP, Goldman M, Grabowski G, Packman S, Wilcox WR: Fabry disease, an under-recognized multisystemic disorder: expert recommendations for diagnosis, management, and enzyme replacement therapy. Ann Intern Med 2003;138:338–346.

4 Meikle PJ, Hopwood JJ, Clague AE, Carey WF: Prevalence of lysosomal storage disorders. JAMA 1999;281:249–254.

5 Tanaka M, Ohashi T, Kobayashi M, Eto Y, Miyamura N, Nishida K, Araki E, Itoh K, Matsushita K, Hara M, Kuwahara K, Nakano T, Yasumoto N, Nonoguchi H, Tomita K: Identification of Fabry's disease by the screening of alpha-galactosidase A activity in male and female hemodialysis patients. Clin Nephrol 2005;64:281–287.

6 Ichinose M, Nakayama M, Ohashi T, Utsunomiya Y, Kobayashi M, Eto Y: Significance of screening for Fabry disease among male dialysis patients. Clin Exp Nephrol 2005;9:228–232.

7 Linthorst GE, Hollak CE, Korevaar JC, Van Manen JG, Aerts JM, Boeschoten EW: alpha-Galactosidase A deficiency in Dutch patients on dialysis: a critical appraisal of screening for Fabry disease. Nephrol Dial Transplant 2003;18:1581–1584.

8 Kotanko P, Kramar R, Devrnja D, Paschke E, Voigtländer T, Auinger M, Pagliardini S, Spada M, Demmelbauer K, Lorenz M, Hauser AC, Kofler HJ, Lhotta K, Neyer U, Pronai W, Wallner M, Wieser C, Wiesholzer M, Zodl H, Födinger M, Sunder-Plassmann G: Results of a nationwide screening for Anderson-Fabry disease among dialysis patients. J Am Soc Nephrol 2004;15:1323–1329.

9 Nakao S, Kodama C, Takenaka T, Tanaka A, Yasumoto Y, Yoshida A, Kanzaki T, Enriquez AL, Eng CM, Tanaka H, Tei C, Desnick RJ: Fabry disease: detection of undiagnosed hemodialysis patients and identification of a 'renal variant' phenotype. Kidney Int 2003;64:801–807.

10 Merta M, Reiterova J, Ledvinova J, Poupetová H, Dobrovolny R, Rysavá R, Maixnerová D, Bultas J, Motán J, Slivkova J, Sobotova D, Smrzova J, Tesar V: A nationwide blood spot screening study for Fabry disease in the Czech Republic haemodialysis patient population. Nephrol Dial Transplant 2007;22:179–186.

11 Thadhani R, Wolf M, West ML, Tonelli M, Ruthazer R, Pastores GM, Obrador GT: Patients with Fabry disease on dialysis in the United States. Kidney Int 2002;61:249–255.
12 Fujii H, Takiuchi S, Kawano Y, Fukagawa M: Putative role of asymmetric dimethylarginine in microvascular disease of kidney and heart in hypertensive patients. Am J Hypertens 2008;21:650–656.
13 Shimotori M, Maruyama H, Nakamura G, et al: Novel mutations of the GLA gene in Japanese patients with Fabry disease and their functional characterization by active site specific chaperone. Hum Mutat 2008;29:331.
14 Ishii S, Sakuraba H, Suzuki Y: Point mutations in the upstream region of the alpha-galactosidase A gene exon 6 in an atypical variant of Fabry disease. Hum Genet 1992;89:29–32.
15 Rolfs A, Böttcher T, Zschiesche M, Morris P, Winchester B, Bauer P, Walter U, Mix E, Löhr M, Harzer K, Strauss U, Pahnke J, Grossmann A, Benecke R: Prevalence of Fabry disease in patients with cryptogenic stroke: a prospective study. Lancet 2005;366:1794–1796.
16 Sachdev B, Takenaka T, Teraguchi H, Tei C, Lee P, McKenna WJ, Elliott PM: Prevalence of Anderson-Fabry disease in male patients with late onset hypertrophic cardiomyopathy. Circulation 2002;105:1407–1411.
17 Chimenti C, Pieroni M, Morgante E, Antuzzi D, Russo A, Russo MA, Maseri A, Frustaci A: Prevalence of Fabry disease in female patients with late-onset hypertrophic cardiomyopathy. Circulation 2004;110:1047–1053.
18 Monserrat L, Gimeno-Blanes JR, Marín F, Hermida-Prieto M, García-Honrubia A, Pérez I, Fernández X, de Nicolas R, de la Morena G, Payá E, Yagüe J, Egido J: Prevalence of Fabry disease in a cohort of 508 unrelated patients with hypertrophic cardiomyopathy. J Am Coll Cardiol 2007;50:2399–2403.
19 Pieroni M, Chimenti C, De Cobelli F, Morgante E, Del Maschio A, Gaudio C, Russo MA, Frustaci A: Fabry's disease cardiomyopathy: echocardiographic detection of endomyocardial glycosphingolipid compartmentalization. J Am Coll Cardiol 2006;47:1663–1671.
20 Andrade J, Waters PJ, Singh RS, Levin A, Toh BC, Vallance HD, Sirrs S: Screening for Fabry disease in patients with chronic kidney disease: limitations of plasma alpha-galactosidase assay as a screening test. Clin J Am Soc Nephrol 2008;3:139–145.
21 Elliott PM, Kindler H, Shah JS, Sachdev B, Rimoldi OE, Thaman R, Tome MT, McKenna WJ, Lee P, Camici PG: Coronary microvascular dysfunction in male patients with Anderson-Fabry disease and the effect of treatment with alpha galactosidase A. Heart 2006;92:357–360.
22 Niizuma S, Takiuchi S, Okada S, Horio T, Kamide K, Nakata H, Yoshihara F, Nakamura S, Kawano Y, Nakahama H, Iwanaga Y, Nakatani S: Decreased coronary flow reserve in haemodialysis patients. Nephrol Dial Transplant 2008;23:2324–2328.
23 Tok D, Gullu H, Erdogan D, Topcu S, Ciftci O, Yildirim I, Muderrisoglu H: Impaired coronary flow reserve in hemodialysis patients: a transthoracic Doppler echocardiographic study. Nephron Clin Pract 2005;101:c200–c206.
24 Weidemann F, Breunig F, Beer M, Sandstede J, Turschner O, Voelker W, Ertl G, Knoll A, Wanner C, Strotmann JM: Improvement of cardiac function during enzyme replacement therapy in patients with Fabry disease: a prospective strain rate imaging study. Circulation 2003;108:1299–1301.
25 Pieroni M, Chimenti C, Ricci R, Sale P, Russo MA, Frustaci A: Early detection of Fabry cardiomyopathy by tissue Doppler imaging. Circulation 2003;107:1978–1984.
26 Fellgiebel A, Keller I, Marin D, Müller MJ, Schermuly I, Yakushev I, Albrecht J, Bellhäuser H, Kinateder M, Beck M, Stoeter P: Diagnostic utility of different MRI and MR angiography measures in Fabry disease. Neurology 2009;72:63–68.
27 Weidemann F, Niemann M, Breunig F, Herrmann S, Beer M, Störk S, Voelker W, Ertl G, Wanner C, Strotmann J: Long-term effects of enzyme replacement therapy on Fabry cardiomyopathy: evidence for a better outcome with early treatment. Circulation 2009;119:524–529.
28 Breunig F, Weidemann F, Strotmann J, Knoll A, Wanner C: Clinical benefit of enzyme replacement therapy in Fabry disease. Kidney Int 2006;69:1216–1221.

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ORIGINAL ARTICLE

Possible association of tumor necrosis factor receptor 2 gene polymorphism with severe hypertension using the extreme discordant phenotype design

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The tumor necrosis factor (TNF)-α pathway has a key role in regulating insulin resistance. TNF receptor 2 (TNFR2) is an emerging candidate gene for insulin resistance in essential hypertension. We examined the association of insulin resistance and enhanced TNF pathway with severe hypertension and the association of a microsatellite polymorphism of the TNFR2 gene with severe hypertension. Male severe essential hypertensive patients (HT) with the onset before 60 years of age and with genetic predispositions to hypertension were consecutively enrolled at our outpatient department (*N*=92). Normotensive men (NT) over 50 years of age were randomly registered from the participants in the annual health check program (*N*=78). Patients were selected as HT and NT who met stringent criteria for systolic/diastolic blood pressure (SBP/DBP) levels ≧180 and/or 110 mm Hg and <120/80 mm Hg, respectively. HT revealed significantly higher plasma insulin levels, C-reactive protein (CRP) and soluble fraction of TNFR2 concentrations (sTNFR2) than NT. A microsatellite polymorphism of the CA repeat in intron 4 of the TNFR2 gene was analyzed. The allele frequency of CA16 in HT differed significantly from that in NT (66/184 *vs.* 36/156, *P*=0.01 by χ^2 analysis). In HT, the CA16 carriers showed significantly higher SBP and plasma insulin levels and a higher tendency of sTNFR2 than did those without this allele. In NT, CA16 carriers revealed significantly higher sTNFR2 and CRP levels than did the CA16 non-carriers. These results suggest that the TNFR2 gene locus has a potential effect on developing severe hypertension through the augmented TNF pathway and insulin resistance.
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Keywords: extreme discordant phenotypes; insulin resistance and C-reactive protein; soluble tumor necrosis factor receptor 2

INTRODUCTION

Concern has been increasing about the association between essential hypertension and metabolic disorders because hypertension occasionally leads to cardiovascular complications.[1] Accumulating evidence suggests that atherosclerosis is derived from metabolic disorders and accompanies the activation of inflammation.[2,3] Chronic subclinical inflammation is associated with arterial stiffness in essential hypertension[4,5] and precedes its onset.[6] Tumor necrosis factor (TNF)-α, once regarded as a cytokine involved in the regulation of the immune system, is now known to participate in insulin resistance.[7,8] TNF-α is hypothesized to have a key role in the pathophysiology of insulin resistance associated with essential hypertension.

Tumor necrosis factor-α expresses its various effects through the binding of two types of TNF receptors, TNFR1 (p55) and TNFR2 (p75).[9] TNFR1 signals universal activities of TNF-α, whereas TNFR2 mainly signals metabolic actions.[10] Circulating soluble TNFR2 (sTNFR2) is shed by proteolytic cleavage from the cell surface and is proposed to buffer TNF-α on the metabolic pathway.[11] sTNFR2 is considered to block TNF-α activity at high concentrations, but to preserve TNF activity by stabilizing its long-term activity at low concentrations. The ratio of sTNF-α receptors (sTNFR2/sTNFR1) has recently been reported to correlate with systolic and diastolic blood pressure levels (SBP and DBP, respectively). Increased circulating sTNFR2, but not sTNFR1 or TNF-α, concentrations were reportedly associated with insulin resistance in healthy volunteers, lean nondiabetic offspring of diabetic patients, and young obese patients.[8,10] Therefore, the sTNFR2 concentration reflects the degree of activation of the TNF pathway and is a surrogate marker of insulin resistance.

We showed that impaired insulin sensitivity is associated with the offspring of essential hypertensives.[12] Whole-body insulin resistance accompanies cellular insulin resistance in immortalized lymphoblasts derived from young, lean hypertensive patients.[13] These previous findings indicate that insulin resistance associated with essential hypertension results from a genetic abnormality. The polymorphisms

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in the 3′-untranslated region of the TNFR2 gene (*TNFRSF1B*) were also linked with obesity, leptin concentrations and insulin resistance in type 2 diabetic patients.[14] Glenn *et al.*[15] reported that a haplotype including a microsatellite marker consisting of 16 CA repeats in intron 4 of the TNFR2 gene was associated with genetic hypertension and higher plasma sTNFR2 concentrations in the case–control study. However, the same group refuted the association of the microsatellite polymorphism with essential hypertension in a later larger-scale study, although the statistical significance is marginal.[16] In this situation, an earlier probability for the association of the microsatellite in the TNFR2 gene with essential hypertension can be estimated.

The false-positive report probability is a formidable issue to accomplish a case–control association study with a small sample size.[17] To raise both the sensitivity and the specificity of a genetic association study, we have been advocating the importance of selective genotyping with extreme discordant phenotypes (EDPs).[13,18] The statistical power of association using the EDP design is estimated as more than double when the sum of the upper and lower selection areas was 0.2.[19,20] Another advantageous procedure is to design a study with a relatively high prior probability (P=0.01–0.25).[17] Enrolling appropriate patients whose intermediate phenotypes are suitable with the pathophysiological pathway relevant to the candidate gene also contributes to raising prior probability and reducing false-positive report probability. In this study, we investigated the relationship between insulin resistance and the TNF pathway and the association of the CA repeat polymorphisms in the TNFR2 gene with severe hypertension. Here, we propose a novel application of a case–control association study with a small sample size to the analysis of a relatively high prior probability using the EDP design.

METHODS

Study population

Hypertensive patients (HT) were consecutively recruited among outpatients between 2000 and 2003 at Keio University Hospital. Recruitment was confined to patients who met all the following criteria: pretreatment for SBP≧180 mm Hg and/or DBP≧110 mm Hg, at least one hypertensive parent, male gender, and onset of hypertension before 60 years of age. BP measurement was performed according to the method described previously.[12] The proportions of HT receiving calcium channel blockers, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, and thiazide diuretics were 74, 65 and 25%, respectively. Patients with diabetes, or heart, renal or endocrinological disease were excluded. Secondary hypertension was excluded by clinical symptoms and physical and laboratory examinations including routine blood chemistry and adrenal function, if necessary. Healthy volunteers were also consecutively recruited among individuals undergoing an annual health examination at Keio University. Normotensive men (NT) who met the following criteria were randomly registered: SBP < 120 mm Hg and DBP < 80 mm Hg; male gender, age over 50 years, and no familial history of hypertension among their first-degree relatives. Patients with acute intercurrent illnesses and chronic inflammatory diseases were also excluded. With these BP levels and genetic predispositions to hypertension, HT and NT were assumed to have extreme phenotypes of genetic hypertension that are consistent with less than 5 and 10% of the general population at their age, respectively.[21,22] This study was conducted according to the principles expressed in the Declaration of Helsinki. The Ethics Review Committee of Keio University School of Medicine approved the protocol. Each patient gave informed consent in writing before enrollment.

Genotyping

Genotyping was performed only in the registered HT and NT. Leukocyte DNA was extracted for genotyping by PCR. The following primers were used: forward, 5′-GTGATCTGCAAGATGAACTCAC-3′ labeled at the 5′-end with 6-FAM; reverse, 5′-ACACCACGTCTGATGTTTCA-3′. Each 20-μl aliquot of PCR mix contained 10 ng of genomic DNA, 100 pmol of each primer, a 2.5 mmol l^{-1} concentration of each dNTP, 0.5 U of TaKaRa Ex Taq DNA polymerase (TaKaRa Bio, Tokyo, Japan), 100 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl (pH 8.0) and 20 mmol l^{-1} $MgCl_2$. After initial incubation at 95 °C for 12 min, 35 cycles were carried out at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, followed by 15 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min; then 20 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and finally incubation at 72 °C for 30 min. The PCR products were electrophoresed using an ABI 3700 automated sequencer (Applied Biosystems, Foster City, CA, USA), and genotypes were assigned using ABI Genotyper software. Alleles were visualized as 263-, 265-, 267-, 269-, 271-, 273- and 275-bp PCR products. The seven alleles were termed CA11, CA12, CA13, CA14, CA15, CA16 and CA17, respectively, to indicate the number of CA repeats.

Plasma assays

Plasma concentrations of TNF-α and sTNFR2 were measured using a Quantikine HS human TNF-α immunoassay and a Quantikine human sTNFR2 immunoassay (R&D Systems, Minneapolis, MN, USA), respectively. Plasma C-reactive protein (CRP) concentrations were measured using an N High Sensitivity CRP kit (Dade Behring, Marburg, Germany). All other blood determinations were performed by using routine clinical methods as previously described.[13]

Statistical analysis

Allele frequencies between the genotype groups and the proportions of the major allele carriers in the TNFR2 gene were assessed by using the χ^2-test for Hardy–Weinberg equilibrium. χ^2 analysis followed by Fischer's exact test was used to estimate the contribution of the microsatellite polymorphism in the TNFR2 gene to HT. We then examined the association of the major alleles (CA15, CA16) with HT. Non-parametric approaches were adopted to compare the biomarkers because the distributions of all the biomarkers were right skewed and the Kolmogorov–Smirnov statistic indicated that some of the distributions of the biomarkers were not normal even after logarithmic transformation. The Mann–Whitney test was used to compare the biomarkers between HT and NT. Correlations between clinical parameters were assessed by Spearman's method in HT and in NT, separately. Next, the Mann–Whitney test was also performed to compare the biomarkers between CA16 carriers and CA16 non-carriers in HT and between CA16 carriers and CA16 non-carriers in NT, separately. Statistical analyses were performed using the SPSS statistical program (SPSS V15, SPSS Inc, Chicago, IL, USA).

RESULTS

Comparisons of clinical parameters between HT and NT

Clinical characteristics of HT and NT are summarized in Table 1. Body mass index (BMI) and waist-to-hip ratio were significantly greater in HT than in NT. Fasting plasma glucose, immunoreactive insulin levels and homeostasis model assessment of insulin resistance index (HOMA-R) were significantly higher in HT than in NT, whereas hemoglobin A1c levels were similar between the groups. Triglyceride concentrations were significantly higher in HT than in NT, and high-density lipoprotein-cholesterol concentrations were significantly lower in HT than in NT. Uric acid concentrations were significantly higher in HT than in NT, whereas plasma sodium, potassium, creatinine and urea nitrogen levels were similar between groups (data not shown). These results clarify that HT were more insulin resistant than NT. White blood cell count and high sensitivity CRP concentrations were significantly higher in HT than in NT. Plasma TNF-α concentrations were significantly lower in HT than in NT; however, sTNFR2 levels were significantly higher in HT than in NT. These results indicate that HT showed augmented inflammatory process through the TNF pathway.

Correlations between clinical parameters

In HT, BMI was significantly correlated with SBP, DBP, HOMA-R, immunoreactive insulin and CRP levels (r=0.241, P<0.05; r=0.267,

Table 1 Clinical characteristics of hypertensive patients and normotensive controls

	Hypertensive patients (N=92)	*Normotensive controls (N=78)*	*P-value by Mann–Whitney*
Clinical characteristics			
Age (years)	48–55–60	53–55–60	0.22
Systolic BP (mm Hg)	170–182–192	104–109–117	<0.0001
Diastolic BP (mm Hg)	110–112–123	66–71–75	<0.0001
Heart rate (beats min^{-1})	68–70–79	65–69–75	0.02
Body mass index ($kg\,m^{-2}$)	24.1–25.3–26.8	20.9–22.2–23.9	<0.0001
Waist-to-hip ratio	0.88–0.91–0.92	0.85–0.88–0.91	0.02
Metabolic parameters			
Glucose (mg per 100 ml)	101–110–122	90–96–104	<0.0001
IRI ($U\,ml^{-1}$)	5.3–9.0–13.0	2.6–3.2–4.1	<0.0001
HOMA-R	1.4–2.5–3.6	0.62–0.76–1.1	<0.0001
Hemoglobin A1c (%)	4.7–5.0–5.2	4.7–4.8–5.1	0.53
TC (mg per 100 ml)	175–206–225	189–210–222	0.40
LDL-C (mg per 100 ml)	92–120–137	102–126–139	0.03
Triglyceride (mg per 100 ml)	95–131–194	62–84–155	<0.0001
HDL-C (mg per 100 ml)	43–50–53	51–58–68	<0.0001
Uric acid (mg per 100 ml)	6.1–6.5–7.2	5.3–6.0–6.5	<0.0001
Inflammatory factors			
WBC count ($\times 10^3/mm^3$)	5.2–5.9–6.1	4.6–5.3–6.1	<0.0001
CRP ($ng\,ml^{-1}$)	0.35–0.66–1.62	0.20–0.28–0.61	<0.0001
TNF-α ($pg\,ml^{-1}$)	1.3–1.8–2.5	1.8–2.3–3.0	0.001
Soluble TNFR2 ($ng\,ml^{-1}$)	1.62–1.85–2.53	1.42–1.57–1.72	<0.0001

Abbreviations: BP, blood pressure; CRP, high-sensitivity C-reactive protein; IRI, immunoreactive insulin; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; HOMA-R, homeostasis model assessment of insulin resistance index; NS, not significant; TC, total cholesterol; TNF, tumor necrosis factor; TNFR2, tumor necrosis factor type 2 receptor; WBC, white blood cell.
Data are expressed as 25 percentile—median—75 percentile.

$P<0.01$; $r=0.266$, $P<0.05$; $r=0.271$, $P<0.01$; and $r=0.264$, $P<0.05$, respectively). Similarly, HOMA-R was significantly correlated with sTNFR2 concentrations in HT ($r=0.381$, $P<0.01$). In NT, BMI was significantly correlated with SBP, DBP, HOMA-R and immunoreactive insulin levels ($r=0.389$, $P<0.0001$; $r=0.290$, $P<0.01$; $r=0.608$, $P<0.0001$; and $r=0.587$, $P<0.0001$, respectively). Similarly, HOMA-R was significantly correlated with CRP levels and sTNFR2 concentrations in NT ($r=0.229$, $P<0.05$ and $r=0.423$, $P<0.0001$, respectively). Therefore, both in HT and NT, BP is related to insulin resistance, which is tightly linked to altered TNF pathway.

TNFR2 gene polymorphisms and insulin resistance

Seven alleles (CA11 to CA17) were identified in the CA repeats of the microsatellite marker in intron 4 of the TNFR2 gene in which genotype frequencies satisfied the Hardy–Weinberg equilibrium. The allele frequencies in HT significantly differed from those in NT ($P=0.01$, χ^2 analysis followed by Fischer's exact test) (Table 2). The common alleles were CA15 and CA16. The allele frequencies of CA15 and CA16 in HT differed markedly from those in NT (CA15, 76/184 *vs.* 85/156, $P=0.02$ by χ^2 analysis, and CA16, 66/184 *vs.* 36/156, $P=0.01$ by χ^2 analysis). We focused on the relationship between CA16 and HT. The odds ratio having the CA16 allele of HT compared with NT is calculated as 52*48/30*40=2.08 (95% confidence interval 1.12–3.85). Clinical characteristics of CA16 allele carriers and CA16 non-carriers are compared in HT and NT, separately. In HT, SBP, HOMA-R, immunoreactive insulin and low-density lipoprotein-cholesterol levels were significantly higher in CA16 carriers than in CA16 non-carriers ($P<0.001$, $P<0.001$, $P<0.01$ and $P<0.05$, respectively) and sTNFR2 showed a similar tendency ($P=0.1$). In NT, CRP and sTNFR2 levels were significantly higher in CA16 carriers than in CA16 non-carriers ($P<0.05$ and $P<0.05$, respectively), although plasma TNF-α concentrations were similar between the groups.

Table 2 Allele frequency of the CA repeats in intron 4 of the TNFR2 gene in hypertensive patients and normotensive controls

CA repeats	*Hypertensive patients (N=92, 184 alleles)*	*Normotensive controls (N=78, 156 alleles)*	*P-value*
CA11	18 (10%)	11 (7%)	
CA12	2 (1%)	2 (1%)	
CA13	3 (2%)	1 (1%)	
CA14	7 (4%)	9 (6%)	0.01
CA15	76 (41%)	85 (54%)	
CA16	66 (36%)	36 (23%)	
CA17	12 (7%)	12 (8%)	

Abbreviation: TNFR2, tumor necrosis factor type 2 receptor.
P-value was assessed by χ^2 analysis followed by Fischer's exact test.

DISCUSSION

In this study, we confirmed that insulin resistance and the augmented TNF pathway are tightly linked with severe hypertension with genetic predispositions. In the TNF pathway, sTNFR2 is of clinical importance in insulin resistance as well as in inflammation. We also showed that the CA16 microsatellite polymorphism in intron 4 of the TNFR2 gene is significantly associated with severe hypertension in a small sample case–control study using the EDP design. The CA16 microsatellite polymorphism in the TNFR2 gene is likely to be associated with higher sTNFR2 concentrations and higher CRP levels in NT. These findings suggest that the TNFR2 gene locus has a potential effect on contributing to the enhanced TNF pathway and insulin resistance, which could develop severe hypertension accompanied by atherosclerosis through an augmented inflammatory process. Several studies have indicated that insulin resistance, in association with essential hypertension, has a genetic component.[12,13,23] However, only a few studies have successfully shown that a mutation is associated with phenotypes that are related to insulin resistance and associated with hypertension.[24–27] This study indicates that the CA16 microsatellite polymorphism in the TNFR2 gene is a potential marker for severe hypertension associated with insulin resistance.

As not only monocytes but also adipocytes express TNF-α, involvement of the TNF-α pathway has been suspected in the pathogenesis of hypertension, dyslipidemia and insulin resistance. Earlier studies have shown that a higher concentration of TNF-α is associated with type 2 diabetes mellitus[28] and that TNF-α production by adipose tissue is accelerated in obesity.[29] Recent studies have indicated that plasma TNF-α is unstable and that sTNFR2 levels represent a more sensitive marker of TNF pathway activity.[11,28] Their findings for sTNFR2 are consistent with this study. However, our finding that the TNF-α levels were lower in HT than in NT is inconsistent with the earlier studies. This might be attributable to the instability of TNF-α measurement and the reciprocal influence of the higher sTNFR2 levels in HT. TNF-α markedly upregulates the expression of TNFR2 mRNA at the lower concentrations. TNFR2 is thought to mediate signaling by membrane-bound ligands in immune and endothelial cells.[30] When TNF-α binds to TNF receptors, the receptor's N-terminal extracellular domain is shed to produce soluble TNF receptors. These receptors inhibit TNF

effects at high concentrations of TNF-α but increase the long-term effects of TNF-α at lower concentrations by sequestering TNF to its membrane receptors. The balance between TNF-α and the sTNF receptors apparently has an important role in the regulation of the TNF pathway. Recent studies have reported that TNFR2 is significantly correlated with insulin resistance, HOMA-R and BP.[10,31] BMI and HOMA-R were significantly greater in HT than in NT. Even if we restricted the inclusion criteria to BMI less than 28 kg m^{-2}, the results were similar (data not shown). Therefore, higher sTNFR2 levels are likely to represent an augmented TNF pathway and inflammatory process, which lead to insulin resistance and severe hypertension.

We readily acknowledge that with so many false-positive reports having been published in genetic association studies, small numbers of the patients are frequently criticized. Although we cannot deny the statistical possibility of a type I error, a significant relationship between the genotype and the series of biologically related intermediate phenotypes supports our hypothesis. Although the process of estimating the prior probability includes subjectivity, the results of one positive case–control study and another larger marginal study indicate the prior probability of this situation as around 0.1, not as 0.01, according to the examples Wacholder *et al.* presented.[15–17] As the odds ratio was 2.08 in this study, false-positive report probability is estimated as less than 0.5, which is considered as a borderline threshold (power=0.3). How have we overcome the borderline threshold of false-positive report probability despite the small sample size? The association study using the EDP design could enhance the statistical power more than double when both the upper and lower selection areas were less than 10 percentile.[18–20] Therefore, the power of this study is estimated as >0.6. Furthermore, although the probability of misclassification between case and control is an annoying issue in measuring phenotypes with large variations such as BP, the EDP design will decrease the possibility of misclassification. In addition, the allele frequency of CA16 is higher in our study than in the Speir study (30 *vs.* 23%). Higher allele frequency is advantageous to detect statistically marginal significance. A weak point of this study lies in the process of sampling NT. Although we randomly selected age- and sex-matched NT who satisfied stringent criteria for the BP level (SBP <120 and DBP <80 mm Hg) and the negative genetic predisposition to hypertension in the same University Hospital, some uncontrolled biases might have influenced the results. BMI was not matched between HT and NT because we focused on the EDP design according to BP levels and the genetic predispositions to hypertension. It remains unclear whether the observed association of the CA16 microsatellite polymorphism of the TNFR2 gene with HT was because of the direct effect of this polymorphism on BP or the indirect effects through confounding factors including the insulin resistance and the inflammation. Although our observation implied that the latter interpretation was more likely, this issue should be clarified in future studies. In some genes, the length of CA repeats, especially in intron 1, may have a role on gene expression and pathogenesis of genetic diseases.[32,33] We cannot rule out the possibility that other genes in linkage disequilibrium with CA16 might be components of severe hypertension through the disturbed TNF pathway and insulin resistance. Our findings are limited to male severe hypertension, whereas it is extremely difficult to enroll female patients using the EDP design at a similar age. Menopause substantially influences blood pressure regulation. Therefore, we should investigate the association using other polymorphisms of the TNFR2 gene and other populations in the future.

In conclusion, insulin resistance is associated with severe hypertension, possibly through an altered TNF pathway. The CA16 microsatellite polymorphism of the TNFR2 gene is a potential marker of severe hypertension with insulin resistance. A case–control association study using the EDP design is an efficient approach to re-explore the possible genetic association.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 1991; **14**: 173–194.
2 Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; **340**: 115–126.
3 Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; **352**: 1685–1695.
4 Mahmud A, Feely J. Arterial stiffness is related to systemic inflammation in essential hypertension. *Hypertension* 2005; **46**: 1118–1122.
5 Pietri P, Vyssoulis G, Vlachopoulos C, Zervoudaki A, Gialernios T, Aznaouridis K, Stefanadis C. Relationship between low-grade inflammation and arterial stiffness in patients with essential hypertension. *J Hypertens* 2006; **24**: 2231–2238.
6 Wang TJ, Gona P, Larson MG, Levy D, Benjamin EJ, Tofler GH, Jacques PF, Meigs JB, Rifai N, Selhub J, Robins SJ, Newton-Cheh C, Vasan RS. Multiple biomarkers and the risk of incident hypertension. *Hypertension* 2007; **49**: 432–438.
7 Yu YH, Ginsberg HN. Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. *Circ Res* 2005; **96**: 1042–1052.
8 Fernandez-Real JM, Ricart W. Insulin resistance and chronic cardiovascular inflammatory syndrome. *Endocr Rev* 2003; **24**: 278–301.
9 Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 1995; **95**: 2409–2415.
10 Fernandez-Real JM, Broch M, Ricart W, Casamitjana R, Gutierrez C, Vendrell J, Richart C. Plasma levels of the soluble fraction of tumor necrosis factor receptor 2 and insulin resistance. *Diabetes* 1998; **47**: 1757–1762.
11 Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J Exp Med* 1992; **175**: 323–329.
12 Ohno Y, Suzuki H, Yamakawa H, Nakamura M, Otsuka K, Saruta T. Impaired insulin sensitivity in young, lean normotensive offspring of essential hypertensives: possible role of disturbed calcium metabolism. *J Hypertens* 1993; **11**: 421–426.
13 Morii T, Ohno Y, Hirose H, Kawabe H, Ogata T, Hirao K, Eguchi T, Maruyama T, Kanno Y, Hayashi M, Saito I, Saruta T. Cellular insulin resistance in Epstein-Barr virus-transformed lymphoblasts from young insulin-resistant Japanese men. *Metabolism* 2005; **54**: 370–375.
14 Fernandez-Real JM, Vendrell J, Ricart W, Broch M, Gutierrez C, Casamitjana R, Oriola J, Richart C. Polymorphism of the tumor necrosis factor-alpha receptor 2 gene is associated with obesity, leptin levels, and insulin resistance in young subjects and diet-treated type 2 diabetic patients. *Diabetes Care* 2000; **23**: 831–837.
15 Glenn CL, Wang WY, Benjafield AV, Morris BJ. Linkage and association of tumor necrosis factor receptor 2 locus with hypertension, hypercholesterolemia and plasma shed receptor. *Hum Mol Genet* 2000; **9**: 1943–1949.
16 Speirs HJL, Wang WYS, Benjafield AV, Morris BJ. No association with hypertension of CLCNKB and TNFRSF1B polymorphisms at a hypertension locus on chromosome 1p36. *J Hypertens* 2005; **23**: 1491–1496.
17 Wacholder S, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J Natl Cancer Inst* 2004; **96**: 434–442.
18 Ohno Y, Tanase H, Nabika T, Otsuka K, Sasaki T, Suzawa T, Morii T, Yamori Y, Saruta T. Selective genotyping with epistasis can be utilized for a major quantitative trait locus mapping in hypertension in rats. *Genetics* 2000; **155**: 785–792.
19 Van Gestel S, Houwing-Duistermaat JJ, Adolfsson R, van Duijn CM, Van Broeckhoven C. Power of selective genotyping in genetic association analyses of quantitative traits. *Behav Genet* 2000; **30**: 141–146.
20 Zhang G, Nebert DW, Chakraborty R, Jin L. Statistical power of association using the extreme discordant phenotype design. *Pharmacogenet Genomics* 2006; **16**: 401–413.
21 Lida M, Ueda K, Okayama A, Kodama K, Sawai K, Shibata S, Tanaka S, Keijnkai T, Horibe H, Minowa M, Yanagawa H, Hashimoto T. Impact of elevated blood pressure on mortality from all causes, cardiovascular diseases, heart disease and stroke among

Japanese: 14 year follow-up of randomly selected population from Japanese—Nippon data 80. *J Hum Hypertens* 2003; **17**: 851–857.
22 Tozawa M, Oshiro S, Iseki C, Sesoko S, Higashiuesato Y, Tana T, Ikemiya Y, Iseki K, Fukiyama K. Family history of hypertension and blood pressure in a screened cohort. *Hypertens Res* 2001; **24**: 93–98.
23 Weidmann P, Bohlen L, de Courten M. Insulin resistance and hyperinsulinemia in hypertension. *J Hypertens Suppl* 1995; **13**: S65–S72.
24 Ikegami H, Yamato E, Fujisawa T, Hamada Y, Fujioka Y, Rakugi H, Higaki J, Murakami H, Shimamoto K, Ogihara T. Analysis of candidate genes for insulin resistance in essential hypertension. *Hypertens Res* 1996; **19** (Suppl 1): S31–S34.
25 Poch E, Giner V, Gonzalez-Nunez D, Coll E, Oriola J, de la Sierra A. Association of the G protein beta3 subunit T allele with insulin resistance in essential hypertension. *Clin Exp Hypertens* 2002; **24**: 345–353.
26 Yamamoto J, Kageyama S, Nemoto M, Sasaki T, Sakurai T, Ishibashi K, Mimura A, Yokota K, Tajima N. PPARgamma2 pro12Ala polymorphism and insulin resistance in Japanese hypertensive patients. *Hypertens Res* 2002; **25**: 25–29.
27 Olivier M, Hsiung CA, Chuang LM, Ho LT, Ting CT, Bustos VI, Lee TM, De Witte A, Chen YD, Olshen R, Rodriguez B, Wen CC, Cox DR. Single nucleotide polymorphisms in protein tyrosine phosphatase 1beta (PTPN1) are associated with essential hypertension and obesity. *Hum Mol Genet* 2004; **13**: 1885–1892.
28 Nophar Y, Kemper O, Brakebusch C, Englemann H, Zwang R, Aderka D, Holtmann H, Wallach D. Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO J* 1990; **9**: 3269–3278.
29 McCarron RM, Wang L, Siren AL, Spatz M, Hallenbeck JM. Monocyte adhesion to cerebromicrovascular endothelial cells derived from hypertensive and normotensive rats. *Am J Physiol* 1994; **267**: H2491–H2497.
30 Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003; **3**: 745–756.
31 Fernandez-Real JM, Lainez B, Vendrell J, Rigla M, Castro A, Penarroja G, Broch M, Perez A, Richart C, Engel P, Ricart W. Shedding of TNF-alpha receptors, blood pressure, and insulin sensitivity in type 2 diabetes mellitus. *Am J Physiol Endocrinol Metab* 2002; **282**: E952–E959.
32 Agarwal AK, Giacchetti G, Lavery G, Nikkila H, Palermo M, Ricketts M, McTernan C, Bianchi G, Manunta P, Strazzullo P, Mantero F, White PC, Stewart PM. CA-Repeat polymorphism in intron 1 of HSD11B2: effects on gene expression and salt sensitivity. *Hypertension* 2000; **36**: 187–194.
33 Brandt B, Meyer-Staeckling S, Schmidt H, Agelopoulos K, Buerger H. Mechanisms of egfr gene transcription modulation: relationship to cancer risk and therapy response. *Clin Cancer Res* 2006; **12**: 7252–7260.

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Experimental Physiology – *Research Paper*

Dihydropyridine- and voltage-sensitive Ca^{2+} entry in human parathyroid cells

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Patch-clamp and fluorescence measurements of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were performed to directly detect extracellular Ca^{2+} entry into cultured parathyroid cells from patients with secondary hyperparathyroidism. Cells loaded with fluo-3 AM or fluo-4 AM showed a transient increase in fluorescence (Ca^{2+} transient) following 10 s exposure to 150 mM K^+ solution in the presence of millimolar concentrations of external Ca^{2+}. The Ca^{2+} transient was completely inactivated after 30–40 s exposure to the high-K^+ solution, was reduced by dihydropyridine antagonists and was enhanced by FPL-64176, an L-type Ca^{2+} channel agonist. The electrophysiological and pharmacological properties of the whole-cell Ca^{2+} and Ba^{2+} currents were similar to those of L-type Ca^{2+} channels. The Ca^{2+} transients induced by 10 s exposure to 3.0 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) were inhibited by dihydropyridine antagonists and were partly inactivated following 30–40 s exposure to the high-K^+ solution. These results demonstrate, for the first time, that human parathyroid cells express L-type-like Ca^{2+} channels that are possibly involved in the $[Ca^{2+}]_o$-induced change in $[Ca^{2+}]_i$. This Ca^{2+} entry system might provide a compensatory pathway for the negative feedback regulation of parathyroid hormone secretion, especially in hyperplastic conditions in which the Ca^{2+}-sensing receptor is poorly expressed.

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The parathyroid gland plays a central role in the tight control of serum Ca^{2+} concentration ($[Ca^{2+}]_o$) by precisely regulating the secretion of parathyroid hormone (PTH). The PTH elevates $[Ca^{2+}]_o$ via its effects on the bones, kidneys and small intestine. Secretion of PTH is increased when $[Ca^{2+}]_o$ is lowered experimentally and vice versa (Brown, 1991; Brown & MacLeod, 2001). Since the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured bovine parathyroid cells changes in parallel with $[Ca^{2+}]_o$, it would appear that the $[Ca^{2+}]_i$ level is the major determinant of PTH secretion (Shoback *et al.* 1984; Nemeth & Scarpa, 1986).

It is generally believed that information relating to $[Ca^{2+}]_o$ is translated into $[Ca^{2+}]_i$ via the Ca^{2+}-sensing receptor (CaR), a G protein-coupled seven-transmembrane-spanning protein that is abundantly expressed in the surface membrane of parathyroid cells (Brown *et al.* 1993; Garrett *et al.* 1995) and other cell types (Brown & MacLeod, 2001). One possible mechanism for the translation of $[Ca^{2+}]_o$ into $[Ca^{2+}]_i$ is the regulation of Ca^{2+} release from intracellular Ca^{2+} stores. In support of this are the findings that inositol 1,4,5-trisphosphate (IP_3) production is increased in bovine parathyroid cells in the presence of relatively high $[Ca^{2+}]_o$ and that HEK 293 cells gain the ability to exhibit IP_3-induced Ca^{2+} release when transfected with a plasmid carrying the cDNA for CaR (Kifor *et al.* 1997).

In addition to the IP_3-mediated pathway, several studies have suggested the involvement of dihydropyridine-sensitive Ca^{2+} channels in the regulation (negative

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feedback) of PTH secretion (Fitzpatrick *et al.* 1986, 1988; Bogin, 1987; Pocotte *et al.* 1995; Wynne *et al.* 1995) and $[Ca^{2+}]_i$ (Chang *et al.* 1995; Pocotte *et al.* 1995; but also see Ridefelt *et al.* 1996; Chang *et al.* 2001). However, definitive evidence that supports the existence of such an extracellular-Ca^{2+}-entry pathway is lacking. Since CaRs are poorly expressed in parathyroid cells from patients with secondary hyperparathyroidism both in humans (reduced immunostaining, Kifor *et al.* 1996; Yano *et al.* 2000) and in rats (reduced immunostaining, Ritter *et al.* 2001; reduced mRNA, Lewin *et al.* 2002), a compensatory or alternative pathway might be prominent in these preparations. The aim of the present study was to directly detect dihydropyridine-sensitive Ca^{2+} entry in human parathyroid cells.

Methods

Cell preparation

The study followed procedures that are in accordance with the Declaration of Helsinki of 1983 and was approved by the Ethics Committee of The Jikei University School of Medicine. Written informed consent was obtained from all patients prior to initiating the surgical operation. In all cases, the removal of hyperplastic parathyroid glands was part of a routine surgical approach and was not related to the goals of this study.

Cultured parathyroid cells were prepared by slight modifications of procedures originally described for the isolation and culture of bovine parathyroid cells (Brown *et al.* 1976). Parathyroid glands were surgically removed from patients with secondary hyperparathyroidism and stored for 2–6 h in cold (<4°C) culture medium [Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (SAFC Biosciences, Brooklyn, Australia), 1 mM sodium pyruvate (Sigma), and penicillin/streptomycin (100 U ml^{-1}, GIBCO BRL)]. Excess connective tissue and fat were removed from the parathyroid glands in ice-cold Hepes-buffered solution containing (mM): NaCl, 146; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 0.5; and Hepes, 10, adjusted to pH 7.4 with NaOH. The parathyroid glands were then minced finely (< 1 mm fragments) with scissors in Hepes-buffered solution containing 2 mg ml^{-1} collagenase (Sigma type IA). Digestion was carried out for 60–70 min in a water bath shaker (130–140 Cycles min^{-1}, TAITEC, Nagoya, Japan) at 37°C, with vigorous pipetting 30–45 min after starting digestion. The turbid solution was filtered through nylon mesh (100 μm) and sedimented for 10 min in a centrifuge (KN-70, Kubota, Japan) at 150–200 ***g***. For patch-clamping and fluorescence recording, isolated cells were plated on sterilized gelatin-coated glass coverslips (14 mm diameter, 0.08–0.12 mm thickness; Matsunami Glass, Osaka, Japan) in culture medium and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 36–74 h. Cell viability, as determined by Trypan Blue exclusion, was routinely around 90%. We confirmed that the cells we used were indeed parathyroid cells by demonstrating structural characteristics (Brown *et al.* 1976) and a transient increase in fluo-3 or fluo-4 fluorescence upon 10 s exposure to exogenous 2.5–3.0 mM Ca^{2+}, 10–100 μM La^{3+} or 5–10 mM Mg^{2+} (Brown 1991). Cells from different patients showed essentially the same response, in that the high-K^+-induced Ca^{2+} transient was abolished and substantially increased by 10 μM dihydropyridines (nitrendipine and nicardipine) and FPL-64176, respectively. We therefore did not investigate the correlation between the severity of secondary hyperparathyroidism and the activity of extracellular Ca^{2+} entry, since that was not the aim of this study. In addition, the serum level of intact PTH was 180.9 ± 13.1 pg ml^{-1} for 175 patients undergoing regular renal replacement therapy. The 175 patients included 63 women and 112 men (average age, 61.5 ± 0.75 years; mean dialysis duration, 10.2 ± 0.57 years). The serum levels of $1,23(OH)_2D_2$, total Ca^{2+}, P_i and total Mg^{2+} were 9.0 ± 0.8 pg ml^{-1}, 9.3 ± 0.05 mg ml^{-1}, 5.2 ± 0.1 mg ml^{-1} and 2.40 ± 0.02 mg ml^{-1}, respectively. Portions of the parathyroid glands used in this study were obtained from each of these patients.

Fluorescence measurements

Changes in $[Ca^{2+}]_i$ were estimated with a Ca^{2+}-sensitive fluorescent indicator dye. Cultured parathyroid cells were loaded for 40–60 min with either fluo-3 AM or fluo-4 AM at a final concentration of 10 μM (Invitrogen, Carlsbad, CA, USA) at 37°C in standard bath solution containing (mM): NaCl, 146; $CaCl_2$, 1.5 or 2.0; KCl, 5; $MgCl_2$, 1; and Hepes, 10, adjusted to pH 7.4 with NaOH. Fluo-4 AM was used, particularly when basal fluorescence intensity was low (e.g. in the presence of 1.5 mM $[Ca^{2+}]_o$), since fluo-4 fluorescence intensity is substantially brighter than that of fluo-3 owing to greater absorption near 488 nm compared with fluo-3 (Gee *et al.* 2000). In order to accelerate dye loading, one volume of pluronic acid (Molecular Probes, Eugene, OR, USA) was added to five volumes of dye-dissolved dimethyl sulphoxide before final dilution. Preliminary experiments confirmed that the baseline fluorescence emitted from either fluo-3 AM- or fluo-4 AM-loaded cells in 1.2 mM $[Ca^{2+}]_o$ was reduced to <15% in the presence of 30 mM of the acetoxymethyl ester form of BAPTA in nominally Ca^{2+}-free saline. Thus, >85% of the fluorescence signal reflected $[Ca^{2+}]_i$. Furthermore, we confirmed that both fluo-3 AM and fluo-4 AM *per se* were essentially non-fluorescent. Fluorescence was detected with a modified Nipkow laser