Table 1. *Continued*

	Weight Loss Maintenance	Rebound	Slow Weight Loss	Weight Loss Resistance
Caloric intake (×1,000kcal/d)				
Entry	2.3 ± 0.6	2.4 ± 0.5	2.4 ± 0.5	2.3 ± 0.4
6 months	1.5 ± 0.4**	1.5 ± 0.3**	1.6 ± 0.3**	1.6 ± 0.2**
12 months	1.4 ± 0.5**	1.5 ± 0.4**	1.5 ± 0.3**	1.5 ± 0.3**
18 months	1.5 ± 0.4**	1.5 ± 0.5**	1.6 ± 0.5**	1.6 ± 0.2**
24 months	1.5 ± 0.3**	1.5 ± 0.4**	1.5 ± 0.4**	1.5 ± 0.2**
Physical activity (×1,000steps/d)				
Entry	9.7 ± 4.3	10.1 ± 4.8	9.6 ± 3.7	9.8 ± 2.9
6 months	21.5 ± 3.8**	22.3 ± 4.1**	20.9 ± 2.9**	20.5 ± 2.1**
12 months	22.5 ± 2.9**	19.5 ± 4.5#	20.4 ± 3.9**	20.1 ± 2.5**
18 months	21.7 ± 2.3**	20.1 ± 3.2**	19.8 ± 3.4**	20.9 ± 2.5**
24 months	20.7 ± 3.7**	19.7 ± 4.1**	22.3 ± 4.5**	20.7 ± 3.0**

Weight loss resistance indicates the subjects who fail to lose weight significantly during 24 months.
BMI = body mass index; BP = blood pressure.
* $P < .05$, † $P < .01$ compared with values in weight loss resistant subjects; ‡ $P < .05$, § $P < .01$ compared with rebound subjects; ‖ $P < .05$, ¶ $P < .01$ compared with values in slow weight loss subjects; # $P < .05$, ** $P < .01$ compared with values at baseline. Data are mean ± SD. $n = 154$.

Statistical Analyses

Genotype frequencies and the Hardy-Weinberg equilibrium were estimated with χ^2 test. Values are shown as mean ± SD. All data analyses were performed with SPSS 8.0 for Windows program (Chicago, IL, USA). Changes in measured parameters within each group and differences among groups were examined by two-way analysis of variance. When these differences were significant, the Dunnett test was used to determine whether the differences of the mean measured variables at 6, 12, and 24 months were significant within the groups and among the groups compared from baseline. Multiple linear regression analyses were used to examine relations among variables using changes in body weight or in mean BP versus changes in hormonal measurements during weight and BP changes.

Results

Prevalence of Weight Loss Maintenance, Rebound Weight Gain, and Weight Loss Resistance

When significant weight loss was defined as a 10% or more reduction in BMI from baseline, 73 subjects succeeded in achieving weight loss at 6 months. Maintenance of weight loss was noted in 37 subjects and rebound weight gain was found in 36 subjects. Sixty other subjects, who did not have a significant weight loss at 6 months, actually succeeded in significant weight loss at 24 months (slow weight loss group). Thus, a total of 97 subjects succeeded in significant weight loss at 24 months. Fifty-seven subjects failed to have significant weight loss at 24 months, 36 subjects had rebound weight gain, and 21 subjects failed to lose weight during the entire 24-month period. Thus, there were four study groups: subjects who failed to lose weight during 24 months represented the weight loss resistant group (n = 21); subjects with maintenance of weight loss represented the weight loss maintenance group (n = 37); subjects with weight regain represented the weight rebound group (n = 36); and those who failed to lose weight at 6 month but succeeded to lose weight at 24 months represented the slow weight loss group (n = 60).

Calorie Intake and Physical Activity

Diet compliance (calorie intake) and physical activity (steps per day) were not significantly different among the four groups (Table 1). Behavior (alcohol intake, cigarette smoking) and socioeconomic status was similar among the four study groups throughout the study. Thus, compliance was considered very good for the 24-month period.

Frequencies of β2- and β3-Adrenergic Receptor Polymorphism

Table 2 shows the frequencies of the genotypes and the alleles of β2- and β3-adrenoceptor genes in the four study groups according to the response in weight loss. The weight loss resistant group, the rebound weight gain group, and the slow weight loss group had a significantly higher frequency of the Gly16 allele of the Arg16Gly of the β2-adrenoceptor compared to the weight loss maintenance group (χ^2 = 5.76, P = .016; χ^2 = 5.38, P = .020; χ^2 = 6.11, P = .013, respectively). The weight loss resistant and slow weight loss groups (both groups failed to lose weight at 6 months) had a higher frequencies of the Glu27 allele of the Gln27Glu of the β2-adrenoceptor compared to a combined group with weight loss maintenance and rebound weight gain group (both groups succeeded in significant weight loss at 6 months) (χ^2 = 6.16, P = .013; χ^2 = 6.22, P = .013, respectively) (Table 2). The frequency distribution of the Glu27 allele of Gln27Glu was 9.4% and that of the Arg64 allele of Trp64Arg

Table 2. Frequencies of the genotype and the allele of the β2- and β3-adrenoceptor polymorphisms in the four study groups according to the response in weight loss

Groups	Genotype (%)			χ^2 Test Among Four Genotype	Allele (%)		χ^2 Test Among Two Alleles
Arg16Gly of β2-adrenoceptor	Arg16/Arg16	Arg16/Gly16	Gly16/Gly16		Arg16	Gly16	
Weight loss maintenance group	18 (48.6%)	14 (37.8%)	5 (13.5%)		50 (67.6%)	24 (32.4%)	
Rebound weight gain group	8 (22.2%)	18 (50.0%)	10 (27.8%)	χ^2 = 13.88, P = .031	34 (47.2%)	38 (52.8%)	χ^2 = 11.36, P = .010
Slow weight loss group	13 (21.7%)	32 (53.3%)	15 (25.0%)		58 (48.3%)	62 (51.7%)	
Weight loss resistant group	3 (14.3%)	12 (57.1%)	6 (28.6%)		18 (42.9%)	24 (57.1%)	
Gln27Glu of β2-adrenoceptor	Gln27/Gln27	Gln27/Glu27	Glu27/Glu27		Gln27	Glu27	
Weight loss maintenance group	33 (91.7%)	4 (10.8%)	0 (0.0%)		70 (94.6%)	4 (5.4%)	
Rebound weight gain group	34 (94.4%)	2 (5.6%)	0 (0.0%)	χ^2 = ---, P = ---	70 (97.2%)	2 (2.8%)	χ^2 = 9.86, P = .020
Slow weight loss group	44 (73.3%)	16 (26.7%)	0 (0.0%)		104 (86.7%)	16 (13.3%)	
Weight loss resistant group	14 (66.7%)	7 (33.3%)	0 (0.0%)		35 (83.3%)	7 (16.7%)	
Trp64Arg of β3-adrenoceptor	Trp64/Trp64	Trp64/Arg64	Arg64/Arg64		Trp64	Arg64	
Weight loss maintenance group	26 (70.2%)	10 (27.0%)	1 (2.7%)		62 (83.8%)	12 (16.2%)	
Rebound weight gain group	28 (77.8%)	8 (22.2%)	0 (0.0%)	χ^2 = 7.91, P = .245	64 (88.9%)	8 (11.1%)	χ^2 = 5.43, P = .143
Slow weight loss group	32 (53.3%)	27 (45.0%)	1 (1.7%)		91 (75.8%)	29 (24.2%)	
Weight loss resistant group	13 (61.9%)	8 (38.1%)	0 (0.0%)		34 (81.0%)	8 (19.0%)	

The definitions for the 4 study groups according to the response in weight loss are referred to in the Results, Prevalence of weight loss maintenance, rebound weight gain, and weight loss resistance section.

was 18.5%. The frequency distributions for alleles in our subjects were similar to those in previous studies in Japanese cohorts, but lower than studies in whites.[23,24]

Physical Measurements

The mean age, BMI, BP levels, and heart rates at entry were similar among the four groups (Table 1). However, the entry measurements for total body fat mass and waist-to-hip ratio were significantly lower in the weight loss maintenance group versus the other three groups (weight loss resistant, slow weight loss, or rebound weight gain). At 6 months, the weight loss maintenance group had significantly greater weight loss, body fat loss, and a decrease in the waist-to-hip ratio compared to the weight loss resistant and slow weight loss groups. The BP reductions at 24 months were significantly greater in the weight loss maintenance and slow weight loss groups compared to the weight loss resistant group and the rebound weight gain group (Table 1). Only in the weight loss maintenance group did the heart rates decline at 24 months.

The subjects carrying the Gly16 allele had greater total body fat mass and waist-to-hip ratios at entry and throughout the study (Table 3), and the subjects carrying the Glu27 allele had greater total body fat mass (Table 4).

In all subjects, weight loss and mean BP reduction during 24 months were 8.9 ± 4.4 kg (10.8% ± 5.3%) and 4.5 ± 3.1 mm Hg (4.7% ± 3.2%). Mean BP reductions per amount of weight lost were similar among the four study groups (0.4 ± 0.2 mm Hg/kg in the weight loss maintenance group; 0.3 ± 0.1 mm Hg/kg in the rebound weight gain group; 0.5 ± 0.2 mm Hg/kg in the weight loss resistant group; and 0.5 ± 0.3 mm Hg/kg in the slow weight loss group).

Hormone Levels

Plasma NE and leptin levels, and HOMA-IR decreased with weight loss in the four study groups (Table 1). The most significant finding was that plasma NE and leptin levels were substantially greater in the weight loss resistant group compared to the weight loss maintenance group at entry and throughout the study. In the rebound weight gain group, plasma NE level was significantly greater than in the weight loss maintenance group. The slow weight loss group also had higher plasma NE and leptin levels at entry compared to the groups who succeeded in a significant weight loss at 6 months (weight loss maintenance and rebound weight gain groups), but lower values than the weight loss resistant group (Table 1). Plasma NE and leptin levels in the subjects carrying the Gly16 and Glu27 alleles were higher at entry and throughout the study compared to those without the Gly16 or Glu27 allele. The HOMA-IR in the subjects with the Gly16 allele was higher throughout the study, as previously we reported,[25] whereas that in the subjects with the Glu27 allele was similar (Tables 3 and 4).

Table 3. Characteristics of subjects according the genotype of the Gly16 at entry and during a weight loss program

Genotype	Without Gly16 Allele (Arg16Arg)			With Gly16 Allele (Arg16Gly + Gly16Gly)		
	At Entry	At 6 Months	At 24 Months	At Entry	At 6 Months	At 24 Months
Subjects (n)	42	42	42	112	112	112
Age (yr)	36 ± 7	37 ± 7§	38 ± 7§	37 ± 6	37 ± 6§	39 ± 6§
BMI (kg/m^2)	27.3 ± 2.0	23.8 ± 2.1	23.6 ± 2.0†§	27.4 ± 1.8	24.5 ± 2.3§	24.6 ± 1.9§
Total body fat mass (kg)	24.0 ± 5.4*	20.2 ± 4.8*‡	17.6 ± 4.5*§	25.5 ± 4.8	21.7 ± 4.3‡	19.2 ± 5.1§
Waist-to-hip ratio	1.16 ± 0.12*	1.05 ± 0.10‡	0.96 ± 0.13*§	1.24 ± 0.10	1.09 ± 0.12‡	1.04 ± 0.14§
Systolic BP (mm Hg)	132 ± 9	130 ± 10	126 ± 9‡	133 ± 10	132 ± 10	131 ± 9
Diastolic BP (mm Hg)	79 ± 9	79 ± 8	75 ± 6‡	79 ± 10	78 ± 9	75 ± 7‡
Mean BP (mm Hg)	97 ± 10	96 ± 10	92 ± 7‡	97 ± 11	96 ± 9	94 ± 9
Heart rate (beats/min)	69 ± 9	67 ± 7	64 ± 7‡	69 ± 7	68 ± 7	66 ± 7
Norepinephrine (pmol/mL)	1.85 ± 0.39*	1.56 ± 0.33*‡	1.26 ± 0.37*§	2.11 ± 0.35	1.80 ± 0.36‡	1.41 ± 0.40§
Leptin (ng/mL)	8.6 ± 2.9*	6.6 ± 2.7*‡	4.9 ± 2.1*§	9.3 ± 3.0	7.3 ± 2.8‡	5.7 ± 2.9‡
HOMA-IR	2.2 ± 0.7*	2.1 ± 0.4	1.8 ± 0.6‡	2.6 ± 0.6	2.3 ± 0.5	2.0 ± 0.5‡

Data are mean ± SD.

BMI = body mass index; BP = blood pressure; HOMA-IR = the homeostasis model assessment of insulin resistance.

* $P < .05$, † $P < .01$ compared with values in subjects with the Gly16 allele; ‡ $P < .05$, § $P < .01$ compared with values at entry.

Relationship With Weight Loss and BP Reduction

Using linear regression analysis, plasma NE levels at entry and at 24 months correlated significantly with mean BP (r = 0.54, P < .001, r = 0.42, P < .001, respectively), heart rate (r = 0.27, P < .05, r = 0.21, P = not significant, respectively), BMI (r = 0.28, P < .05, r = 0.25, P < .05, respectively), total body fat mass (r = 0.36, P < .001, r = 0.35, P< .001, respectively), and plasma leptin level (r = 0.42, P < .001, r = 0.37, P < .001, respectively). Changes in heart rate for 24 months did not correlate with changes in plasma NE.

In multiple linear regression analysis, total body fat mass (P = .043), plasma NE (P = .016) and leptin levels (P = .020), but not heart rate, at entry were significant determinant factors for absolute weight changes for 24 months (R^2 = 0.337, F = 3.56, P = .010). Mean BP (P = .050), total body fat mass (P = .041), and plasma NE level (P = .042) at entry were significant determinant factors for absolute changes in mean BP for 24 months (R^2 = 0.301, F = 2.45, P = .047). Changes in total body fat mass (P = .019), waist-to-hip ratio (P = .034), plasma NE (P = .033) and leptin levels (P = .022) for 2 years were significant determinant factors for absolute changes in mean BP (R^2 = 0.381, F = 5.03, P = .007).

Discussion

The present study shows that the initial levels of total body fat mass, plasma NE and leptin levels, and the frequency of the Gly16 allele of the Arg16Gly of the β2-adrenoceptor polymorphism are significantly higher in people resistant to weight loss and those who have rebound weight gain compared to those with successful weight loss maintenance. Thus, measurement of these parameters might predict those subjects who will fail to lose weight in both the short and long term or who will regain weight after an initial success in weight loss as determined in a dietary and exercise weight loss program. In addition, the frequency of the Glu27 allele of the β2-adrenoceptor is higher in subjects who are weight loss resistant and in those with slow weight loss, in people who fail to lose weight in the short term (6 months), compared to those with weight loss maintenance or rebound weight gain, who lose weight in the short term. These findings indicate that sympathetic overactivity as reflected by high plasma NE levels associated with the Gly16 and Glu27 polymorphisms might be linked to mechanisms that explain weight loss resistance and rebound weight gain despite adherence to long-term diet and exercise programs.

A number of studies have demonstrated several BP-lowering mechanisms accompanying weight loss.[6,7] In our weight loss program, average percent reduction in body weight and mean BP for 24 months in all subjects were 10.8% and 4.7%, respectively. These results revealed similar values to those levels in the meta-analysis by Neter et

Table 4. Characteristics of subjects according the genotype of the Glu27 at entry and during a weight loss program

Genotype	Without Glu27 Allele (Gln27Gln)			With Glu Allele (Gln27Glu)		
	At Entry	At 6 Months	At 24 Months	At Entry	At 6 Months	At 24 Months
Subjects (*n*)	125	125	125	29	29	29
Age (yr)	36 ± 6	37 ± 6§	38 ± 6§	37 ± 7	37 ± 7§	39 ± 7§
BMI (kg/m²)	27.4 ± 1.7	24.0 ± 2.1§	24.1 ± 2.0§	27.3 ± 1.8	24.9 ± 2.2‡	24.5 ± 2.1§
Total body fat mass (kg)	24.5 ± 5.7*	20.5 ± 4.5*§	18.0 ± 4.3*§	25.9 ± 6.0	22.4 ± 3.8‡	20.1 ± 4.7§
Waist-to-hip ratio	1.19 ± 0.13	1.09 ± 0.10	0.98 ± 0.12§	1.22 ± 0.11	1.13 ± 0.09	1.03 ± 0.13‡
Systolic BP (mm Hg)	133 ± 9	131 ± 10	125 ± 9*‡	134 ± 10	133 ± 9	131 ± 10
Diastolic BP (mm Hg)	79 ± 9	79 ± 9	75 ± 9‡	79 ± 9	78 ± 7	75 ± 9
Mean BP (mm Hg)	97 ± 10	96 ± 9	91 ± 8*‡	97 ± 11	96 ± 7	94 ± 10
Heart rate (beats/min)	69 ± 8	67 ± 7	65 ± 7	69 ± 7	68 ± 6	65 ± 7
Norepinephrine (pmol/mL)	1.94 ± 0.33*	1.67 ± 0.41*‡	1.29 ± 0.34*§	2.20 ± 0.33	1.92 ± 0.45	1.51 ± 0.42‡
Leptin (ng/mL)	7.1 ± 2.8*	6.9 ± 2.8*	5.1 ± 2.7*‡	9.5 ± 3.1	8.0 ± 3.0‡	5.8 ± 2.8‡
HOMA-IR	2.4 ± 0.4	2.1 ± 0.7	2.0 ± 0.6	2.5 ± 0.6	2.3 ± 0.6	2.0 ± 0.5

Data are mean ± SD.
Abbreviations as in Table 3.
* $P < .05$, † $P < .01$ compared with values in subjects with the Glu27 allele; ‡ $P < .05$, § $P < .01$ compared with values at entry.

al.[7] And, normalization of BP often occurs before obese subjects reach their ideal weight. Therefore, overweight and obese hypertensive patients should be encouraged to lose even a modest amount of weight as it has pronounced beneficial effects on BP levels and other risk factors.

It is established that weight loss is accompanied by reductions in sympathetic nerve activity (SNA), insulin resistance, plasma leptin levels, and BP levels.[1–3,5] However, few investigations have examined how the sympathetic nervous system, insulin resistance, and leptin level are involved in weight loss resistance and rebound weight gain.[26] More than 20 years ago, Tuck et al found significant reductions in SNA and BP during rapid weight loss and weight loss using a very low calorie diet.[2,3,5] In the present study, we note that plasma NE, leptin, and the HOMA-IR levels track with weight changes and in addition that plasma NE and leptin levels at entry are determinant factors for predicting changes in body weight during a weight loss program, thus further demonstrating that SNA (plasma NE levels) and plasma leptin levels are major control factors for changes in body weight.[5,19,27]

In the present study, we used plasma NE levels as an index of SNA. Tuck,[28] Grassi and Esler,[29] and Rahn et al[30] reviewed that there are different results in SNA values in hypertensive patients depending on the method of SNA measurement including: regional NE spillover, muscle sympathetic nerve activity (microneurography), and plasma NE measurements. Spillover methods are considered as the gold standard for SNA measurements, but in humans these are difficult and invasive measurements. Furthermore , Rumantir et al[31] reported different values for regional sympathetic nerve activity between the kidneys and heart in obesity-related hypertensive subjects. Plasma NE levels are more practical for large population studies, [5,15,19,25] but represent several different process (secretion, clearance, and reuptake of NE) making it difficult to determine whether the defect is overproduction or decreased metabolism.

Pathophysiological involvement of genetic abnormalities in the β2- and β3-adrenoceptor system in obesity are well described.[10–15,22] Among β2- and β3-adrenoceptor polymorphisms, amino acid substitutions, Arg16Gly and Gln27Glu of the β2-adrenoceptor and Trp64Arg of the β3-adrenoceptor polymorphism are considered functionally important in the control of body weight.[10–15,22] In the present study, the weight loss maintenance group have a lower frequency of the Gly16 and Glu27 alleles of the β2-adrenoceptor and lower plasma NE levels, suggesting that the Gly16 and Glu27 alleles are related to a blunted β2-adrenoceptor activity and resultant sympathetic overactivity as shown by higher plasma NE levels.[15] Furthermore, the slow weight loss and weight loss resistance groups in our study during a 24-month period have a higher frequency of the Glu27 allele and higher plasma NE levels compared to the groups who succeed in significant weight loss in the short term. We have reported that the individuals carrying the Gly16 and Glu27 alleles have greater weight gain and BP elevations.[15] Taken together,

one could propose that the characteristics of the Gly16 and Glu27 alleles of the β2-adrenoceptor polymorphisms during weight gain may stabilize body weight even with on-going caloric restriction and exercise causing resistance to weight loss.

Kaye et al[32] found a strong relationship between heart rate and the level of cardiac sympathetic nerve activity measured by the spillover method. Our results show that changes in plasma NE do not correlate with changes in heart rate, whereas heart rate correlates with plasma NE at entry. These findings indicate that the limitation that plasma NE level does not always precisely reflect the response of regional (heart) sympathetic nerve activity to weight change, but we could speculate that the subjects carrying the Gly16 or Glu27 alleles who have less reductions in heart rate might have an impaired response of cardiac sympathetic nerve activity to weight loss through the blunted β2-adrenoceptor sensitivity and resultant cardiac risk through resistance to weight loss. However, further studies are needed to evaluate the differences in the sympathetic-mediated thermogenesis in the subjects carrying the β2-adrenoceptor polymorphisms.

In conclusion, greater adiposity and sympathetic overactivity (high plasma NE levels) might predict those obese individuals who have complete resistance to lose weight during the 24-month period and those who will have rebound weight gain after a successful initial weight loss. The sympathetic overactivity in those subjects who have rebound weight gain and in those who have resistance to weight loss may be associated with the polymorphisms in the Gly16 and Glu27 alleles of the β2-adrenoceptor.

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Association of Hypoadiponectinemia With Smoking Habit in Men

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Abstract—Adiponectin is emerging as an important molecule in obesity, the metabolic syndrome, and cardiovascular disease. On the other hand, smoking habit is well known to be related to cardiovascular disease and hypertension. To examine the association between adiponectin concentration and smoking habit, we performed an epidemiological survey and an acute exposure test in humans and an experiment in adipocytes to elucidate the mechanism underlying the association between adiponectin and smoking. In the epidemiological study, we enrolled a total of 331 male subjects to examine chronic smoking exposure. Plasma adiponectin was significantly lower ($P=0.01$) in current smokers (5.3 ± 0.3 μg/mL) than in never-smokers (6.5 ± 0.4 μg/mL). A significant association between smoking and low adiponectin level was also confirmed in multiple regression analysis including age, body mass index, hypertension, diabetes, hyperlipidemia, and creatinine clearance (never-smokers 6.5 ± 0.4 μg/mL; past smokers 5.6 ± 0.3 μg/mL; current smokers 5.2 ± 0.4 μg/mL; $F=4.52$; $P=0.01$). To examine the acute effect of smoking on adiponectin concentration for 12 hours, we measured plasma adiponectin level in 5 male never-smokers before smoking and 3, 6, and 12 hours after smoking, with the result that adiponectin showed a significant decrease after smoking (12 hours; $-14.5\pm0.6\%$; $P<0.01$). In cultured mouse 3T3-L1 adipocytes, H_2O_2 and nicotine reduced the mRNA expression and secretion of adiponectin in a dose-dependent manner. Smoking habit is associated with adiponectin concentration in men, and its suppressive effect is mediated in part through direct inhibition of smoking on adiponectin expression in adipocytes. (***Hypertension*. 2005;45:1094-1100.**)

Key Words: smoking ■ oxidative stress ■ risk factors ■ lipids ■ lipoprotein ■ metabolism

Cigarette smoking exacts a continuing toll on public health and is an established risk factor for hypertension and cardiovascular disease, and nonsmoking is a leading preventive strategy against coronary artery disease. Furthermore, cigarette smoking and its cessation are reported to alter lipid metabolism.[1–3] It is well established that smoking stimulates lipolysis in vivo. The lipolytic effect of smoking has been attributed to the nicotine component being mediated via release of catecholamines.[3] Nicotine, a major component of cigarette smoke, promotes inflammation[4] and progression of atherosclerotic lesions.[5,6] Furthermore, nicotine also has a direct effect on human adipose tissue.[7–9] On the other hand, oxidative stress has been shown to be a key phenomenon involved in the effects of smoking. Cigarette smoke contains a large amount of free radicals, which degrade NO released from the endothelium and also produce highly reactive intermediates, resulting in endothelial injury. Oxidative stress can damage many cell components, such as DNA, lipid membranes, and proteins, and lead to apoptosis and cell damage.[10,11]

Adiponectin, an adipose tissue–specific collagen-like factor, is abundantly present in plasma and possesses antiatherogenic properties. Adiponectin is emerging as an important molecule in obesity,[12] the metabolic syndrome,[13–15] cardiovascular disease,[16] lipid metabolism,[15] and hypertension.[17,18] In addition, adiponectin concentration is correlated independently with the vasodilator response to reactive hyperemia, and its concentration could be an independent parameter of endothelial function.[19] Endothelial dysfunction, an early marker of atherosclerosis, has been observed in chronic smokers as well as after acute cigarette smoking.[20,21] These results suggest that adiponectin may be a mediator between smoking and several diseases such as hypertension and coronary artery disease. Furthermore, smoking may directly regulate adiponectin concentration via lipolysis.

Although Miyazaki et al[22] reported that in subjects with coronary artery disease, smoking status was associated with reduced adiponectin concentration, using a small number of subjects, the association between plasma adiponectin and smoking status was evaluated without adjusting for con-

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founding factors and without consideration of the sex difference in adiponectin level.[23] Sex is an important confounding factor for evaluating adiponectin concentration, and the clinical importance of smoking habit in evaluating adiponectin concentration has not been fully elucidated. In the present study, we examined whether smoking habit is associated with a lower adiponectin level. First, we performed a cross-sectional study using a large number of subjects, including only males, to examine the chronic effect of smoking. Second, we performed an acute smoking exposure test in never-smokers and evaluated the effect for 12 hours. Finally, we demonstrated an inhibitory effect of H_2O_2 and nicotine on the expression and secretion of adiponectin in vitro.

Methods

Epidemiological Study (Chronic Effect of Smoking)

A total of 331 male subjects were selected from patients who were admitted and underwent medical investigation including a general check-up at Osaka University Hospital, Japan. All subjects enrolled in this study were Japanese. The study protocol was approved by the ethical committee of Osaka University, and all subjects gave written informed consent to participate in the study. All procedures followed were in accordance with the institutional guidelines of Osaka University. Smoking status was determined by interview on the day of measuring clinical parameters, and the subjects were divided into 3 groups according to smoking habit: never-smokers, past smokers (who had a history of habitual smoking but had quit), and current smokers. As a result, the numbers of never-smokers, past smokers, and current smokers were 79, 136, and 116, respectively. Hypertension was defined as systolic blood pressure (BP) of ≥140 mm Hg or diastolic BP of ≥90 mm Hg on repeated measurements, or receiving antihypertensive treatment. Diabetes mellitus was defined according to World Health Organization criteria.[24] Hyperlipidemia was defined as total cholesterol (T-chol) of >6.22 mmol/L, triglyceride (TG) of >2.26 mmol/L, or HDL cholesterol (HDL-chol) of <0.91 mmol/L. Ischemic heart disease was defined as a ≥75% organic stenosis of ≥1 major coronary artery, as confirmed by coronary angiography or a history of myocardial infarction or percutaneous transluminal coronary angioplasty. Renal failure was defined as fasting serum creatinine (Cr) concentration >176.8 μmol/L. Subjects with ischemic heart disease, chronic renal failure, nephrotic syndrome, overt congestive heart failure, valvular heart disease, secondary hypertension, or atrial fibrillation were excluded. Furthermore, no subjects receiving steroid therapy were included in this study.

Each subject was studied on the day after admission, in the morning after having abstained from alcohol, caffeine, and smoking, as well as food for 8 hours before the study. BP was measured by well-trained physicians, and venous blood was drawn from all subjects. Height and body weight were measured and body mass index (BMI) calculated. Plasma samples for subsequent assay were stored at −80°C. Insulin sensitivity was estimated using the homeostatic model assessment (HOMA) index (ie, plasma glucose level×(plasma insulin level/22.5). Brinkman index was calculated using the formula: number of cigarettes smoked per day×number of years of smoking. Plasma concentration of adiponectin was determined using a sandwich ELISA system (Adiponectin ELISA kit; Otsuka Pharmaceutical Co. Ltd.), as reported previously.[12] The parameters T-chol, TG, HDL-chol, and Cr levels were also determined. Urine samples were collected for 24 hours to evaluate Cr clearance (Ccr).

Acute Smoking Exposure Test

To examine the acute effect of smoking on adiponectin concentration, we measured plasma adiponectin level in 5 healthy volunteers who had never smoked (age 33 to 46 years; BMI 24.0±1.0 kg/m²). All subjects were male and were coauthors included in this study, and the exclusion criteria of this study were the same as those described previously. After completion of the baseline study, all participants were asked to smoke a cigarette (1.1 mg nicotine; 14 mg tar) and were instructed to inhale. Before and 3, 6, and 12 hours after smoking, venous blood was drawn.

Effect of H_2O_2 and Nicotine on Expression and Secretion of Adiponectin In Vitro

3T3-L1 mouse preadipocytes were grown to confluence and induced to differentiate into adipocytes, as described previously.[25] Seven days after the initiation of differentiation (assessed by this criterion), 85% to 90% of the cells were judged to be differentiated. On day 7, the indicated concentrations of H_2O_2 with/without *N*-acetyl-L-cysteine (NAC) or nicotine (Sigma) were added to the media for 24 hours.

An aliquot of the media after 24 hours of stimulation was subjected to ELISA (Adiponectin ELISA kit; Otsuka Pharmaceutical Co. Ltd.) to detect the amount of adiponectin secreted.

Loss of 3T3-L1 adipocyte integrity was evaluated spectrophotometrically by measurement of lactate dehydrogenase (LDH) activity in the supernatant using a standard kit (LDH-Cyotoxic Test; Wako).

3T3-L1 adipocyte cellular protein samples were isolated using ISOGEN (Nippon Gene) according to manufacturer protocol. Adipocyte protein concentration was determined by colorimetric protein assay (detergent solubilization) using DC Protein Assay (Bio-Rad) according to manufacturer protocol. The relative secretion of adiponectin into the media was normalized to the amount of cellular protein in the same sample.

Total RNA from adipocytes was isolated using ISOGEN, treated with DNAse to prevent contamination with genomic DNA, and finally resuspended in diethylpyrocarbonate-treated MilliQ. Expression levels of adiponectin and 18S mRNA were quantified by real-time quantitative RT-PCR using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Inc.) according to manufacturer instructions. TaqMan probes and primers for adiponectin and 18S were Assay-on-Demand gene expression products (Applied Biosystems, Inc.). We used amplification of 18S ribosomal RNA in each of the stimulated conditions for sample normalization. The relative expression of adiponectin mRNA was normalized to the amount of 18S in the same mRNA sample using the standard curve method described by the manufacturer.

Statistical Analysis

Means or proportions of clinical characteristics and cardiovascular risk factors were computed for each smoking pattern. Continuous variables were expressed as mean±SEM. Differences between smoking status groups for variables including adiponectin concentration were analyzed by 1-way ANOVA and post hoc comparison (Dunnet's procedure). Unpaired *t* test was used to examine the differences in adiponectin between 2 groups. Pearson's correlation coefficients were used to assess the relationships between adiponectin and all other variables. Multiple regression models were used to assess the relationship between adiponectin concentration and smoking status after adjustment for potential confounding factors. The significance of differences in adiponectin levels before and after smoking was evaluated using repeated-measures ANOVA. In the in vitro study, differences were analyzed by unpaired *t* test. All *P* values were 2-sided, and those <0.05 were considered statistically significant. All calculations were performed using a standard statistical package (JMP 4.0; SAS Institute).

Results

Association of Plasma Adiponectin Concentration With Smoking Habit in Humans

The clinical and biochemical characteristics of the study subjects divided into 3 groups according to smoking habit are shown in Table 1. We first examined the association between smoking habit and adiponectin concentration. The concentra-

TABLE 1. Clinical Characteristics of Study Subjects

Variables	Never-Smokers	Past Smokers	Current Smokers
n	79	136	116
Brinkman index	0±0	792±53	742±58
Age, years	58.0±1.2	62.2±0.9*	57.5±1.0
BMI	23.6±0.3	23.7±0.3	23.2±0.3
Adiponectin, μg/mL	6.5±0.4	5.7±0.3	5.3±0.3*
Systolic BP, mm Hg	130±2	134±1	133±2
Diastolic BP, mm Hg	80±1	81±1	85±1*
Hypertension, %	66.7	71.0	73.9
Diabetes, %	10.3	15.9	20.0
Hyperlipidemia, %	27.9	30.0	38.0
T-chol, mmol/L	4.99±0.09	5.18±0.08	5.26±0.10*
TG, mmol/L	1.48±0.12	1.78±0.09	1.64±0.11
HDL-chol, mmol/L	1.48±0.05	1.45±0.04	1.41±0.04
HOMA index	1.7±0.3	2.0±0.3	2.1±0.4
Cr, μmol/L	82.0±2.5	80.6±1.8	76.3±2.2
Ccr, mL/min	85.7±3.7	82.4±2.6	83.5±3.2

Values are given as mean±SEM.
*$P<0.05$ compared with never-smokers for each parameter.

TABLE 2. Clinical Characteristics of Subgroups Without Medication and Diabetes

Variables	Never-Smokers	Past Smokers	Current Smokers
n	27	41	30
Brinkman index	0±0	850±94	554±74
Age, years	58.8±2.5	62.0±2.1	60.1±2.5
BMI	22.6±0.5	22.3±0.4	21.8±0.3
Adiponectin, μg/mL	8.3±0.8	7.1±0.6	6.1±0.7*
Systolic BP, mm Hg	117±4	125±3	128±4
Diastolic BP, mm Hg	74±3	76±2	79±3
Hypertension, %	14.8	17.1	16.1
Hyperlipidemia, %	31.8	29.4	34.8
T-chol, mmol/L	4.99±0.15	5.14±0.15	4.90±0.16
TG, mmol/L	1.49±0.20	1.48±0.18	1.64±0.22
HDL-chol, mmol/L	1.55±0.10	1.53±0.09	1.62±0.11
HOMA index	1.1±0.4	1.4±0.3	1.5±0.7
Cr, μmol/L	72.9±6.7	75.4±4.7	76.6±7.8
Ccr, mL/min	84.0±5.4	80.8±4.1	83.0±5.4

Values are given as mean±SEM.
*$P<0.05$ compared with never-smokers for each parameter.

tion of adiponectin was significantly lower in current smokers than in never-smokers ($P=0.01$). Furthermore, the concentration of adiponectin showed a tendency to be lower in past smokers than in never-smokers ($P=0.06$). Diastolic BP and T-chol in current smokers and age in past smokers were significantly higher than those in never-smokers ($P<0.05$). In addition, the kinds of drugs that influence adiponectin concentration, such as angiotensin II receptor blockers, angiotensin-converting enzyme (ACE) inhibitors, and peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands, were not significantly different among the smoking status.

In the total subjects, adiponectin level was significantly associated with age ($r=0.38$; $P<0.01$), BMI ($r=-0.33$; $P<0.01$), and Ccr ($r=-0.36$; $P<0.01$). Furthermore, adiponectin level was significantly lower in patients with hypertension (5.1±0.2 versus 7.3±0.3 μg/mL; $P<0.01$), diabetes (5.0±0.2 versus 6.2±0.3 μg/mL; $P<0.01$), and hyperlipidemia (4.5±0.3 versus 5.8±0.2 μg/mL; $P<0.01$). We next performed multiple regression analysis including age, BMI, hypertension, diabetes, hyperlipidemia, and Ccr and revealed that adiponectin concentration in never-smokers was ≈1.25× higher than that in current smokers (never-smokers 6.5±0.4 μg/mL; past smokers 5.6±0.3 μg/mL; current smokers 5.2±0.4 μg/mL; $F=4.52$; $P=0.01$).

To exclude the effect of diabetes and drugs on adiponectin concentration, we next examined the effect of smoking habit on adiponectin concentration after excluding subjects with diabetes and subjects receiving any medication. The clinical and biochemical characteristics of these study subjects are shown in Table 2. Adiponectin concentration significantly increased with age ($r=0.41$; $P<0.01$) and HDL-chol ($r=0.43$; $P<0.01$) and decreased with BMI ($r=-0.50$; $P<0.01$), systolic BP ($r=-0.35$; $P<0.01$), diastolic BP ($r=-0.36$; $P<0.01$), TG ($r=-0.30$; $P<0.05$), HOMA ($r=-0.29$; $P<0.05$), and Ccr ($r=-0.41$; $P<0.01$). On the other hand, there was no significant association between adiponectin and T-chol ($r=-0.04$). Although clinical variables other than adiponectin concentration were not significantly different, adiponectin concentration was significantly lower in current smokers than in never-smokers ($P=0.04$).

Brinkman index was not associated with adiponectin concentration in the total subjects ($r=-0.05$) or in subjects without medication or diabetes ($r=-0.19$). However, in current smokers (n=116), the number of cigarettes smoked per day was inversely associated with adiponectin concentration ($r=-0.21$; $P<0.04$).

Effect of Acute Smoking Exposure on Plasma Adiponectin Concentration

The mean adiponectin level before smoking was 7.0±1.5 μg/mL. Percent changes in plasma concentration of adiponectin in response to smoking are shown in Figure 1. Acute smoking exposure produced a significant decrease in plasma level of adiponectin at 3 hours (−9.2±0.7%) and 6 hours (−13.1±1.2%), and the maximum decrease was observed at 12 hours after smoking (−14.5±0.6%; $F=17.3$; $P<0.01$).

Inhibitory Effects of H_2O_2 and Nicotine on Expression and Secretion of Adiponectin in 3T3-L1 Adipocytes

We investigated the effect of H_2O_2 and nicotine on the regulation of adiponectin secretion and gene expression in 3T3-L1 adipocytes. Incubation with H_2O_2 or nicotine reduced adiponectin mRNA expression and adiponectin secretion into the media in a dose-dependent manner (Figures 2 and 3). The effects of H_2O_2 to reduce adiponectin mRNA expression and secretion into the media were antagonized by coincubation with NAC (Figure 2). Secretion of adiponectin into the media was significantly reduced compared with control by nicotine

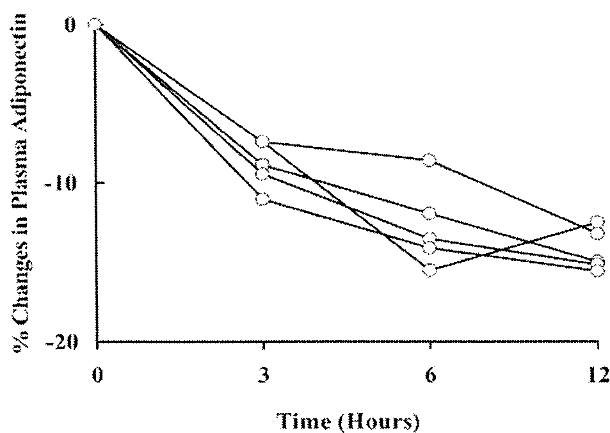

Figure 1. Percent changes in plasma adiponectin levels before and after smoking. Individual changes in adiponectin level were plotted. Adiponectin levels were expressed as percent change from initial values (n=5).

at concentrations $\geq 10^{-8}$ mol/L. We next studied the adipocyte protein concentration; the amount of adiponectin in the media was adjusted by each of the amount of cellular protein. As shown in Figures 2B and 3B, even after adjustment for protein amount, adiponectin secretion was significantly reduced by incubation with H_2O_2 or nicotine in a dose-dependent manner.

Cytotoxicity was also assessed by LDH leakage from adipocytes into the media. As shown in Figure 2C, H_2O_2 (100 μmol/L) significantly increased LDH release from adipocytes. When cultured in the presence of NAC (10^{-2} M), this increase was significantly attenuated. On the other hand, as shown in Figure 3C, treatment with nicotine also significantly increased leakage of LDH from adipocytes at concentrations $\geq 10^{-7}$ mol/L.

Discussion

The present study demonstrated that the plasma adiponectin concentration was significantly lower in male subjects who were current smokers than in never-smokers, and the association was observed even in subjects without diabetes and medication. Furthermore, multiple regression analysis including age, BMI, hypertension, diabetes, hyperlipidemia, and Ccr showed that adiponectin concentration was significantly lower in current smokers. Acute smoking exposure reduced adiponectin concentration significantly at 12 hours after smoking in never-smokers. In cultured 3T3-L1 adipocytes, oxidative stress and nicotine reduced the secretion and expression of adiponectin. These results suggest that smoking may decrease plasma adiponectin concentration in men.

In this study, even in subjects without diabetes and medication, the association between adiponectin concentration and clinical variables was in accordance with previous reports that adiponectin concentration was significantly associated with age,[18,26] BMI,[12] TG,[13] HDL-chol,[27] BP,[18] and insulin resistance indicated by HOMA.[14]

Although adiponectin concentration is decreased in several diseases,[12–14,16,18] the mechanisms that regulate plasma adiponectin concentration have not been fully elucidated. It has been reported that weight reduction[13] and certain drugs such as PPAR-γ ligands,[25] ACE inhibitors, and angiotensin II receptor blockers[28] increased the adiponectin concentration, a cytokine, tumor necrosis factor-α (TNF-α), reduced the expression of adiponectin in adipocytes,[25] and some human mutations of adiponectin affect plasma adiponectin concentration.[18,29] In this study, we demonstrated that smoking habit is also associated with adiponectin concentration. Furthermore, our finding of lower adiponectin levels in chronic smokers is in line with the fact that chronic smokers are insulin resistant.[30] Thus, our results may support investigation of the mechanisms of several disorders induced by smoking.

Smoking is known to be associated with increased oxidative stress. Reactive oxygen species such as H_2O_2 are also normally produced during cellular oxidation reduction processes. Although our results showed significant cytotoxicity in adipocytes incubated with H_2O_2 at a concentration of 100 μmol/L, this cytotoxicity was significantly attenuated when they were cultured with NAC. Furthermore, H_2O_2 decreased the expression and secretion of adiponectin from adipocytes in a dose-dependent manner. Previous reports have shown that oxidative stress disrupts activation of phosphatidylinositol 3-kinase (PI3K),[31,32] which is a key molecule in the secretion of adiponectin in 3T3-L1 adipocytes.[33] Thus, we propose the idea that oxidative stress induced by tobacco smoke decreases the secretion and expression of plasma adiponectin via inhibition of activation of PI3K in adipocytes.

Nicotine activates nicotinic acetylcholine (nACh) receptors, which belong to the family of ionotropic receptors consisting of 5 transmembrane subunits building up ion channels. nACh receptors are widely distributed throughout the central and peripheral nervous system and are involved in signal transmission at the skeletal neuromuscular junction, in autonomic ganglia, and in the brain.[34,35] Functional nACh receptors are expressed in adipocytes in mice,[36] and nicotine exerts direct stimulation of lipolysis via nACh receptors in human adipose tissue.[7–9] Thus, nicotine has the possibility of regulating adiponectin concentration directly. In our experiments, nicotine had a significant inhibitory effect at concentrations $\geq 10^{-7}$ mol/L, which can be found in the plasma of smokers.[37] Furthermore, our results also showed significant cytotoxicity in adipocytes incubated with nicotine at a concentration of 10^{-6} mol/L. These results could also be in accordance with previous reports that nicotine itself induces lipolysis by activating local nicotinic cholinergic receptors in adipose tissue.[7] Thus, our results indicate that nicotine in tobacco smoke decreases plasma adiponectin via inhibition of the secretion and expression of adiponectin in adipocytes.

Apart from nicotine and oxidative stress, there are several other possible mechanisms by which smoking habit may affect adiponectin concentration. It has been reported that smoking itself and tissue hypoxia elevate TNF-α,[38,39] a powerful proinflammatory cytokine and a mediator of inflammation, which is known to decrease adiponectin concentration.[25] These findings also support the idea that persistent production of TNF-α induced by chronic exposure to cigarette smoke may promote the development of hypoadiponectinemia. Furthermore, nicotine elicits release of the catecholamines epinephrine and norepinephrine,[40] and

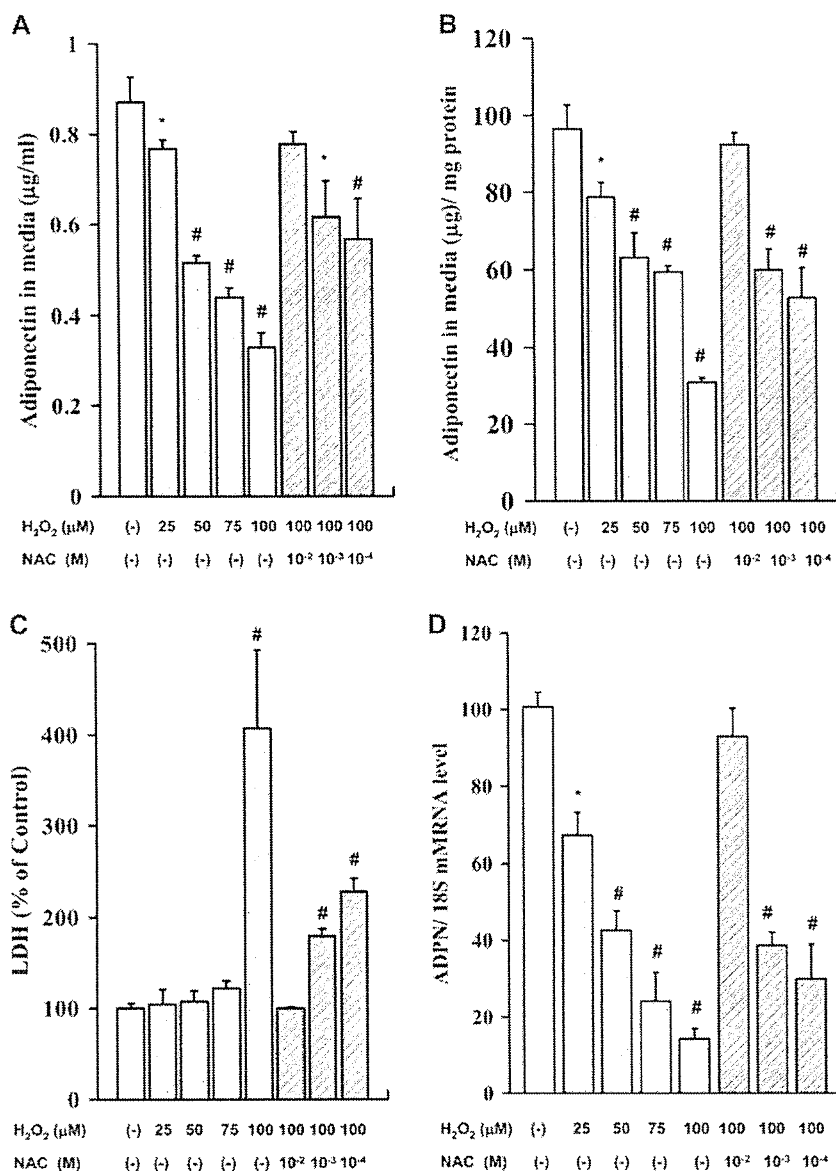

Figure 2. Effects of H_2O_2 on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose-effect of H_2O_2 with/without NAC on adiponectin secreted into media (A), adjusted by the amount of protein (B), LDH leakage (C), and adiponectin mRNA level (D). All LDH leakage and mRNA are plotted as percent change relative to the level with 0 μmol/L H_2O_2 treatment. Values are given as mean±SEM (n=12 in each group). **P*<0.05 and #*P*<0.01 compared with 0 μmol/L H_2O_2 treatment for each variable.

β-adrenergic stimulation suppresses adiponectin gene expression.[41]

With respect to cessation of habitual smoking, in this study, adiponectin level was between those of nonsmokers and current smokers, even after adjustment for confounding factors. These results suggest that the decreasing effect of smoking on adiponectin concentration might remain even after smoking cessation. Another reason is that even after smoking cessation, smoking-related damage persisted, such as endothelial dysfunction and continuing low-grade inflammation indicated by C-reactive protein,[42] which is known to affect adiponectin concentration.[19,43] To clearly confirm whether smoking cessation affects adiponectin concentration, a cohort study is required.

Because tobacco smoke consists of >4000 chemical constituents, it is impossible to predict the effect of nicotine and oxidative stress within this complex mixture of components. Although we showed that nicotine and oxidative stress have a potent inhibitory effect on adiponectin secretion, there are several other molecules in cigarette smoke that may be toxic to adipocytes (eg, cadmium, cotinine, and thiocyanate).[44] The net effect of cigarette smoke on the function of adiponectin may be quite different from that of nicotine or H_2O_2 alone. Another limitation is that this study was designed as a cross-sectional study rather than a randomized clinical trial or observational study. Furthermore, several important determinants of adiponectin level, such as body fat content and waist circumference, were not measured in our study. Instead of these measurements, we included HOMA and BMI in the analysis of this study. Previous reports have shown that body fat content, especially intra-abdominal fat, is a determinant of adiponectin level.[26] On the other hand, the different localization of fat mass itself influences cardiovascular risk factors such as T-chol, TG, and HDL-chol.[45] In our study, except for T-chol, the clinical characteristics were not significantly different among subjects (Table 1). Furthermore, the subjects

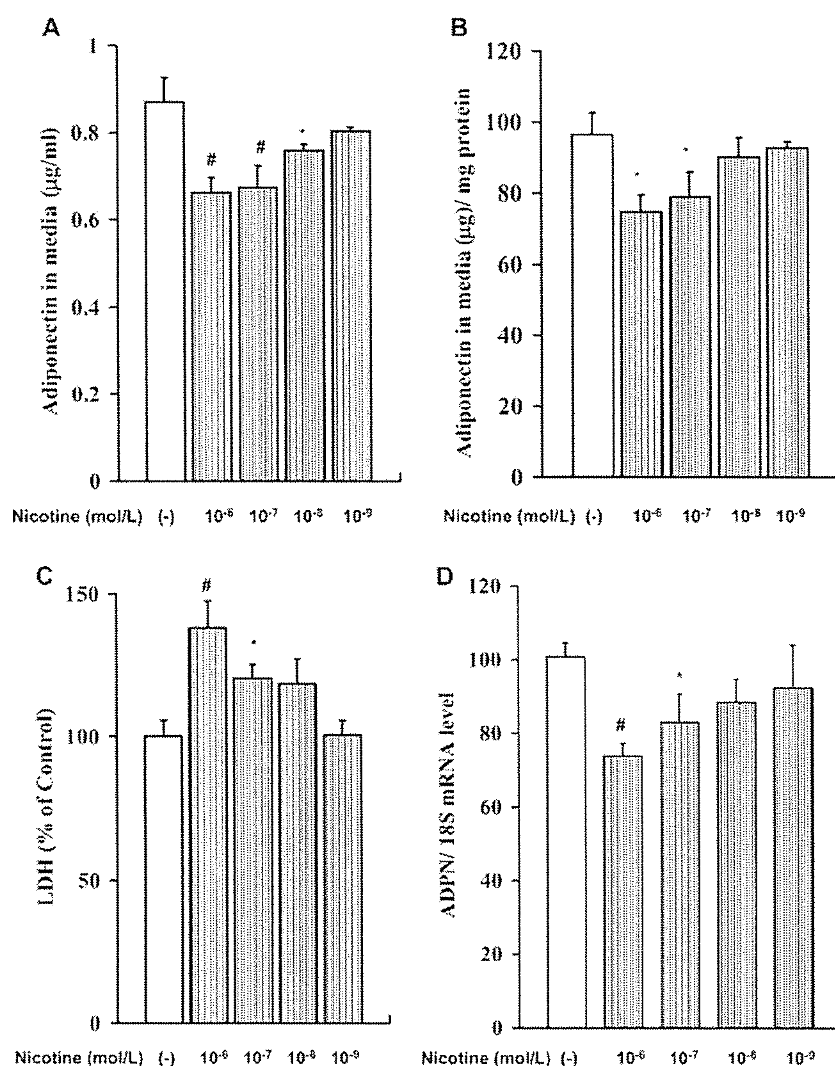

Figure 3. Effects of nicotine on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose-effect of nicotine on adiponectin secreted into media (A), adiponectin adjusted by the amount of protein (B), LDH leakage (C), and adiponectin mRNA level (D). All LDH leakage and mRNA are plotted as percent change relative to the level with 0 mol/L nicotine treatment. Values are given as mean±SEM (n=12 in each group). *$P<0.05$ and #$P<0.01$ compared with 0 mol/L nicotine treatment for each variable.

included in this study were relatively lean, and obesity (BMI $\geq$30 kg/m^2) was present in only 2.5% of the total subjects. Thus, the effect of different fat distributions on adiponectin concentration among the groups may be relatively small in this study. On the other hand, our study could not provide a conclusion on the influence of "passive smoking" on adiponectin concentration. Further investigation is required to examine these effects.

In conclusion, our results demonstrated that smoking habit is associated with a lower adiponectin concentration in men. This reduction may be induced through a direct effect of oxidative stress and nicotine on adipocytes.

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Germline mutations in *HRAS* proto-oncogene cause Costello syndrome

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Costello syndrome is a multiple congenital anomaly and mental retardation syndrome characterized by coarse face, loose skin, cardiomyopathy and predisposition to tumors. We identified four heterozygous *de novo* mutations of *HRAS* in 12 of 13 affected individuals, all of which were previously reported as somatic and oncogenic mutations in various tumors. Our observations suggest that germline mutations in *HRAS* perturb human development and increase susceptibility to tumors.

Costello syndrome (OMIM 218040) is characterized by mental retardation, high birth weight, neonatal feeding problems, curly hair, coarse face, nasal papillomata and loose integuments of the back of the hands (**Fig. 1a,b**)[1,2]. This syndrome phenotypically overlaps with Noonan syndrome (OMIM 163950). Missense mutations in *PTPN11*, encoding tyrosine phosphatase SHP-2, were identified in 50% of affected individuals with Noonan syndrome[3] but not in those with Costello syndrome[4,5]. Mutant SHP-2 proteins identified in Noonan syndrome were gain-of-function mutants with enhanced phosphatase activity, which resulted in activation of a RAS-MAPK (mitogen activated protein kinase) cascade in a cell-specific manner[6,7]. We hypothesized that genes mutated in Costello syndrome and *PTPN11*-negative Noonan syndrome encode molecules that function upstream or downstream of SHP-2 in signal pathways (**Fig. 1c**). Among these molecules, we sequenced the entire coding regions of the four RAS genes[8], *KRAS*, *HRAS*, *NRAS* and the recently identified *ERAS*, in genomic DNA from 13 individuals with Costello syndrome and 28 individuals with *PTPN11*-negative Noonan syndrome (**Supplementary Methods** online).

We identified four heterozygous mutations in *HRAS* in 12 individuals with Costello syndrome: 38G→A (G13D) in two individuals (COS30 and COS44), 34G→A (G12S) in seven individuals (COS35, COS38, COS64–COS66, COS68 and COS70), 35G→C (G12A) in two individuals (COS62 and 69) and 35GC→TT (G12V) in individual COS37 (**Table 1**). Individual COS37 died of severe cardiomyopathy (**Supplementary Fig. 1** online). These mutations have been identified somatically in various tumors[8] (**Fig. 1d,e**). Mutation analysis of genomic DNA from two different tissues in three affected individuals and genomic DNA from parents in four families indicated that these 'oncogenic' and germline mutations occurred *de novo* (**Supplementary Fig. 2** online). None of the above mutations were observed in 100 control chromosomes (data not shown). We observed no mutations in *KRAS*, *NRAS*, *HRAS* or *ERAS* in 28 individuals with Noonan syndrome or in one individual with Costello syndrome.

RAS genes encode 21-kDa proteins that are members of the superfamily of small GTP-binding proteins. Mutations at codons 12 and 13 are in constitutively active GTP-bound conformation and activate downstream effectors such as MAPK, PI-3 kinase and RalGDS[9]. Individuals with Costello syndrome often show hyperkeratosis of the skin, hypertrophic cardiomyopathy or relative macrocephaly, suggesting that the disorder is associated with altered cell growth, proliferation or cell death. Five of the seven affected individuals whom we examined had hypertrophic cardiomyopathy (**Supplementary Table 1** and **Supplementary Fig. 1** online). Cell proliferation studies showed that fibroblasts from four affected individuals had increased uptake of 5-bromodeoxyuridine compared with three independent controls when treated with 1 or 10 ng ml^{-1} epidermal growth factor or 10% fetal calf serum (**Fig. 1f**). These results are indicative of increased growth factor–dependent proliferation, which might partially explain the mechanism of organ hypertrophy in Costello syndrome.

Costello syndrome is often associated with benign tumors, including papillomata, and cancers, including rhabdomyosarcoma, ganglioneuroblastoma and bladder carcinoma[2,10]. The ganglioneuroblastoma of individual COS62 was surgically removed when she was two years and six months of age (**Supplementary Fig. 1** online). We sequenced genomic DNA extracted from the tumor tissue and detected heterozygosity with respect to the wild-type (G) and mutated (C) alleles, excluding the possibility of loss of heterozygosity in this region (**Fig. 1g**). Sequencing of cDNA transcribed from ganglioneuroblastoma mRNA showed monoallelic expression of the mutated allele (C). In contrast, we observed biallelic expression in cDNA from her fibroblasts. This monoallelic expression in tumor, the mechanism of which remains to be elucidated, might be responsible for the pathogenesis of cancer development in Costello syndrome.

Since an oncogenic *HRAS* mutation was first identified in T24/EJ bladder carcinoma[11], mutations of *KRAS*, *HRAS* and *NRAS* have been found in ~30% of human cancers[8]. To the best of our knowledge, Costello syndrome is the first disorder associated with germline mutations in the RAS family of GTPases. Germline mutations in Costello syndrome were detected only in *HRAS*, and not in other RAS

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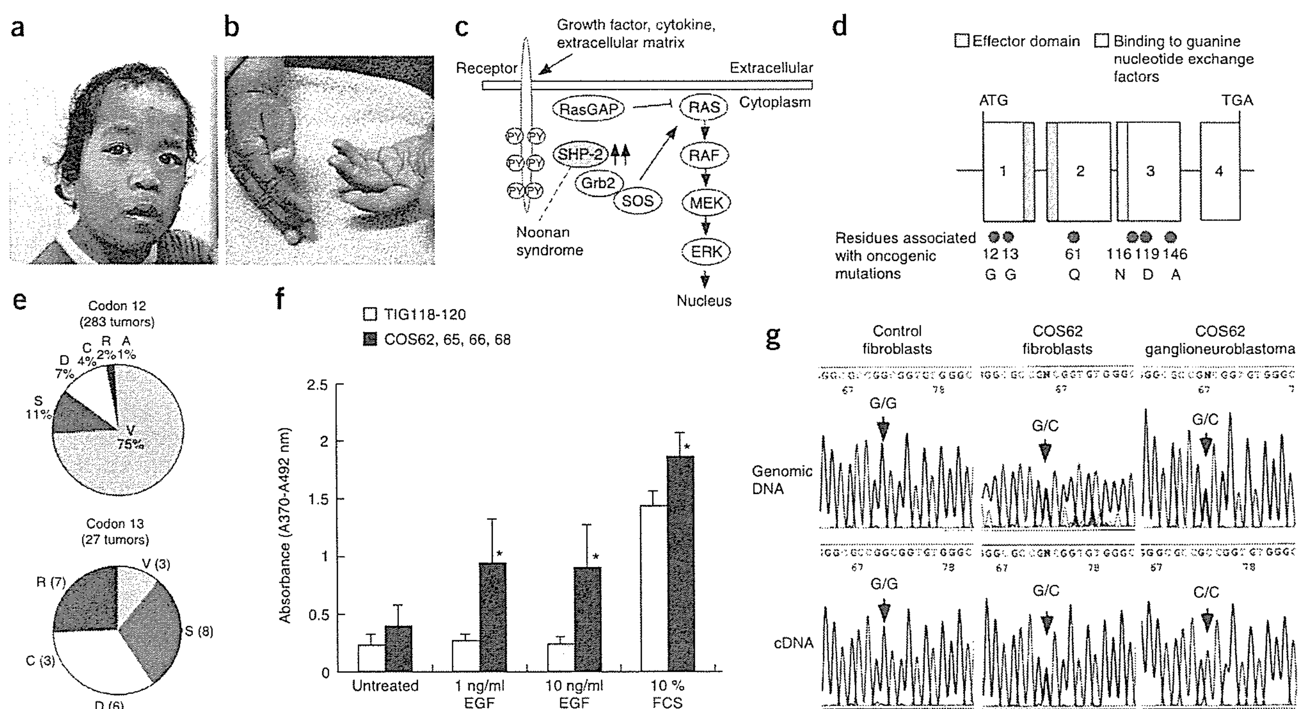

Figure 1 *HRAS* mutations in Costello syndrome. **(a)** Typical facial appearance and **(b)** loose skin and increased creases of the hands in individual COS35. **(c)** Signal transduction pathway including SHP-2 and RAS-MAPK. **(d)** Domain organization and genomic structure of *HRAS*. **(e)** The spectrum and relative frequency of amino acid substitutions at codons 12 and 13 of *HRAS* found in somatic tumors, according to the Sanger Institute Catalogue of Somatic Mutations in Cancer website. Parenthesis showed the number of tumors reported. **(f)** Increased 5-bromodeoxyuridine uptake in fibroblasts derived from affected individuals (black bars) when treated with 1 or 10 ng ml^{-1} epidermal growth factor (EGF) or 10% fetal calf serum (FCS). 5-bromodeoxyuridine uptake was assayed by immunostaining with antibody to 5-bromodeoxyuridine-POD. Absorbance was read at dual wavelengths of 370 and 492 nm. $^*P < 0.05$. Results are expressed as the means ± s.d. from each cell line. TIG118-120, control fibroblasts. **(g)** Monoallelic RNA expression in ganglioneuroblastoma from individual COS62.

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family genes, suggesting that *HRAS* has a distinct role from other RAS molecules in human development. This is in accordance with recent studies reporting embryonic lethality in *Kras*-knockout mice but not in *Hras1*- or *Nras*-knockout mice[9]. It is not known whether SHP-2 transmits its activating signals only to HRAS or to all RAS homologs. The overlapping phenotype between Noonan syndrome and Costello syndrome suggests that the activation of signals is transmitted from SHP-2 at least to HRAS. Increased activation of downstream pathways from HRAS may be a common pathogenic mechanism in Noonan syndrome and Costello syndrome.

The G12V mutant of HRAS had the lowest GTPase activity among various amino acid substitutions at codon 12 (ref. 12). Biological assays by focus formation in NIH3T3 cells or soft agar growth show that the substitution by valine at codon 12 has the highest transformation potential (G12V>G12S, G12A>G13D)[13,14]. Individual COS37 with the G12V mutation died of severe cardiomyopathy at the age of 18 months. The most potent mutation, G12V, is predominant in human cancers (**Fig. 1e**). The germline mutations found in Costello syndrome seem to be less potent in general than the mutations identified in cancer.

Table 1 Mutations in *HRAS* in individuals with Costello syndrome

Individual	Origin	Material used for genotyping	Nucleotide substitution	Amino acid change	Genotype of father/mother
COS30	Japanese	Leukocytes	38G→A	G13D	NA/NA
COS35	Japanese	Leukocytes	34G→A	G12S	NA/NA
COS38	Japanese	Leukocytes, buccal cells	34G→A	G12S	WT/WT
COS62	Japanese	Lymphoblasts, fibroblasts	35G→C	G12A	WT/WT
COS37	Japanese	Leukocytes	35GC→TT	G12V	WT/WT
COS44	Japanese	Leukocytes	38G→A	G13D	NA/NA
COS64	Japanese	Leukocytes, buccal cells	34G→A	G12S	WT/WT
COS65	Italian	Fibroblasts	34G→A	G12S	NA/NA
COS66	Italian	Fibroblasts	34G→A	G12S	NA/NA
COS68	Italian	Fibroblasts	34G→A	G12S	NA/NA
COS69	Italian	Lymphoblasts	35G→C	G12A	NA/NA
COS70	Italian	Lymphoblasts	34G→A	G12S	NA/NA

NA, not available; WT, wild-type.

A tumor screening protocol for those with Costello syndrome has been proposed[10]. *HRAS* mutations alone are not sufficient to transform human cells[15], and oncogenic RAS mutations seem to be one of the genetic events during multistep carcinogenesis. We hope our findings will provide a reliable means of diagnosing Costello syndrome and help to elucidate the precise clinical representations, including cancer development, response to therapy and overall natural history of affected individuals.

URL. The Sanger Institute Catalogue of Somatic Mutations in Cancer website is http://www.sanger.ac.uk/cosmic/.

Accession codes. GenBank: *KRAS* coding region, NC_000012; *HRAS* coding region, NC_000011; *NRAS* coding region, NC_000001; *ERAS* coding region, NC_000023; *HRAS* cDNA, NM_005343.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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ORIGINAL ARTICLE

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Functional analysis of PTPN11/SHP-2 mutants identified in Noonan syndrome and childhood leukemia

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Abstract Noonan syndrome (NS) is characterized by short stature, characteristic facial features, and heart defects. Recently, missense mutations of *PTPN11*, the gene encoding protein tyrosine phosphatase (PTP) SHP-2, were identified in patients with NS. Further, somatic mutations in *PTPN11* were detected in childhood leukemia. Recent studies showed that the phosphatase activities of five mutations identified in NS and juvenile myelomonocytic leukemia (JMML) were increased. However, the functional properties of the other mutations remain unidentified. In this study, in order to clarify the differences between the mutations identified in NS and leukemia, we examined the phosphatase activity of 14 mutants of SHP-2. We identified nine mutations, including a novel F71I mutation, in 16 of 41 NS patients and two mutations, including a novel G503V mutation, in three of 29 patients with leukemia. Immune complex phosphatase assays of individual mutants transfected in COS7 cells showed that ten mutants identified in NS and four mutants in leukemia showed 1.4-fold to 12.7-fold increased activation compared with wild-type SHP-2. These results suggest that the pathogenesis of NS and leukemia is associated with enhanced phosphatase activity of mutant SHP-2. A comparison of

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the phosphatase activity in each mutant and a review of previously reported cases showed that high phosphatase activity observed in mutations at codons 61, 71, 72, and 76 was significantly associated with leukemogenesis.

Keywords Tyrosine phosphatase · SHP-2 · SHP2 · *PTPN11* · Noonan syndrome · Leukemia

Introduction

Noonan syndrome (NS; MIM#163950) is an autosomal dominant developmental disorder characterized by facial dysmorphism including hypertelorism, low-set ears and ptosis, short stature, skeletal abnormalities, and heart defects (Mendez and Opitz 1985; Allanson 1987). Frequently observed features in NS patients are pulmonary stenosis, hypertrophic cardiomyopathy, chest deformities, webbed and short neck, mental retardation, and genitourinary defects including cryptorchidism in males, and bleeding diathesis due to factor XI deficiency (Mendez and Opitz 1985). The incidence of this syndrome is estimated to be 1:1,000 to 1:2,500 live births (Allanson 1987). Tartaglia et al. (2001) have identified missense mutations in *PTPN11*, a gene encoding tyrosine phosphatase SHP-2, in 45% of clinically diagnosed NS patients. Patients with NS have been known to be associated with juvenile myelomonocytic leukemia (JMML), a myeloproliferative disorder characterized by excessive production of myelomonocytic cells (Tartaglia et al. 2003). Interestingly, somatic mutations in *PTPN11* were identified in 34% of JMML cells and in a small percentage of patients with myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and acute lymphoid leukemia (ALL) from non-NS-leukemic patients (Tartaglia et al. 2003; Loh et al. 2004; Tartaglia et al. 2004).

SHP-2, a widely expressed cytoplasmic tyrosine phosphatase, has been implicated in signal transduction pathways elicited by growth factors, cytokines, hormones, and extracellular matrix (Servidei et al. 1998; Feng 1999; Neel et al. 2003). One of the well-known pathways is the activation of the RAS/MAPK (mitogen-activated protein kinase) cascade induced by epidermal, fibroblast, and hepatocyte growth factors (Feng 1999; Neel et al. 2003). SHP-2 contains a tandem array of two SH2 domains at its N terminus, a catalytic domain in the middle, and a C-terminal domain that contains tyrosine phosphorylation sites. A crystallographic analysis indicates that intramolecular conformational change controls its catalytic activity (Hof et al. 1998). In the inactive state, the N-SH2 domain directly binds the PTP domain and blocks its catalytic activity. Once the N-SH2 domain binds phosphotyrosine peptide, conformational change results in the active state. Previously identified mutations in NS and leukemic patients were primarily restricted in or around the interacting face of the N-SH2 and PTP domains (Kosaki et al. 2002; Musante et al. 2003; Sarkozy et al. 2003; Zenker et al. 2004), suggesting that they are gain-of-function mutations with enhanced phosphatase activity. Mutations identified in leukemia and NS occurred at the same amino acid residues, but the type of substitutions rarely overlapped.

Recent studies have shown that the phosphatase activities of five mutations were elevated and that the phosphatase activities of two mutations (D61Y and E76K) identified in JMML cells were higher than that of the N308D mutation identified in NS (Tartaglia et al. 2003; Fragale et al. 2004). In this study, we performed phosphatase assays for 14 mutants identified in NS and leukemia to examine the presence of any functional differences between the mutations. Finally, we examined the functional consequences of the mutations in cultured cells.

Materials and methods

Patients

Forty-one patients with NS were recruited. The diagnosis of NS was evaluated by clinical geneticists based on the cardinal criteria delineated by Allanson (1987); characteristic faces including hypertelorism, down-slanting palpebral fissures and ptosis, webbed or short neck, chest deformity, cubitus vulgas, short stature, congenital heart defects, developmental delay, and cryptorchidism. Thirty-eight patients were sporadic cases, and three were familial cases. Bone marrow cells, peripheral blood, or cell lines were obtained from 29 leukemia cases without NS (seven ALL, 18 AML, one CML, two MDS, and one JMML). Eighteen AML patients were classified according to French-American-British classification (FAB); two with M0, one with M1, five with M2, three with M4, three with M5, one with M7, and three with unknown classification. This study was approved by the Ethics Committee of the Tohoku University School of Medicine.

Mutation analysis

After obtaining written informed consent, genomic DNA was isolated from the patients' peripheral leukocytes, Epstein-Barr virus-transformed lymphoblastoid cells, or leukemia cells. Fifteen coding exons of *PTPN11* from 41 NS patients and exons 3 and 13 from leukemia patients were sequenced. Each *PTPN11* exon with flanking intronic sequences was amplified with primers based on GeneBank sequences (GeneBank accession no. *NT 009775*). The M13 reverse or forward sequence was added to the 5′ end of the polymerase chain reaction (PCR) primers for use as a sequencing primer. The PCR was performed in 30 μl of a solution containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10% (v/v) DMSO, 0.4 pmol of each

primer, 100 ng genomic DNA, and 2.5 U of Taq DNA polymerase. The reaction condition consisted of 35 cycles of denaturation at 94°C for 15 s, annealing at 57°C for 15 s, and extension at 72°C for 30 s. The products were gel-purified and sequenced on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, Fostercity, CA, USA).

Construction of the plasmids

The human SHP-2 cDNA was PCR amplified by using the following primers: the 5′ primer included sequences encoding an *Eco*RI site followed by the FLAG M5 epitope (MDYKDDDDK) (Aoki et al. 2000). In addition, the 3′ primer had an *Eco*RI site. The amplified fragment was digested with *Eco*RI and subcloned into an *Eco*RI site of pBluescript KSII+ (Stratagene). Mutant constructs were generated using a Quickchange site-directed mutagenesis kit (Stratagene). All mutant and normal constructs were verified by sequencing. The full-length SHP-2 cDNA and mutants were digested with *Eco*RI and subcloned into an *Eco*RI site of the pCAGGS expression vector, a mammalian expression vector that has a cytomegalovirus enhancer, and a chicken β-actin promoter (Niwa et al. 1991).

Immune complex tyrosine phosphatase assay

The COS7 cells were purchased from the American Tissue Culture Collection (ATCC). The COS7 cells were maintained in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded at 6×10^5 cells/6-cm dish, and 24 h later, 2.0 µg of pCAGGS vectors encoding one of the wild-type (WT) or mutant SHP-2 cDNAs were transfected using 8 µl of LipofectAMINE reagent and 12 µl of PLUS reagent (Invitrogen, Carlsbad, CA, USA). The E76A mutant identified somatically in leukemia cells (Tartaglia et al. 2003) was used as the positive control in each assay because this mutant was proved to have an increased phosphatase activity in a Xenopus experiment (O'Reilly et al. 2000). Cells were serum starved for 24 h, scraped, and collected by centrifugation after two washes with phosphate buffered saline (PBS). Lysates were prepared in 1 ml ice-cold RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1:100 protease inhibitor, and 1% Triton X) and incubated on ice for 15 min. The lysates were centrifuged at 14,000 *g* for 15 min at 4°C, and 1 mg of protein was used for immunoprecipitation. The FLAG-tagged WT SHP-2, and mutants were immunoprecipitated with anti-FLAG M5 antibody (Sigma, St Louis, Mosby, USA) for 2 h at 4°C. Immune complexes were collected by adding 50 µl of 50% protein G-Sepharose beads slurry (Amersham Biosciences Corp., Piscataway, NJ, USA) for 1 h at 4°C, washed twice with RIPA buffer, and then washed twice with phosphatase assay buffer (20 mM Hepes pH 7.5, 250 mM NaCl, 1 mM EDTA, 1 mM DTT).

Immune complex phosphatase assay was performed at 30°C for 30 min in 25 µl phosphatase assay buffer supplemented with 500 µM Src phosphopeptide (pTSTEPQpYQPGENL). The reaction was linear at the range of 0–1,000 µM Src. After brief centrifugation, supernatants were collected, added to 100 µl Malachite Green solution (Upstate Biotechnology), and incubated for 15 min at room temperature. The absorbance at 620 nm was measured, and the increase in the phosphatase activity was calculated by subtracting the background blank values (negative Src). Values for phosphate release were then obtained by comparing with the standard curve (linear at 0–2,000 pmol of phosphate). In order to measure the level of immunoprecipitated proteins, 50 µl 2× SDS sample buffer was added to the beads and boiled for 3 min. Twenty µl was used for SDS-PAGE followed by immunoblotting with anti-FLAG M5 antibody.

ERK (extracellular signal-regulated kinase) phosphorylation

Human embryonic kidney (HEK) 293 cells (ATCC) were maintained in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 µg/ml of streptomycin. Cells were seeded at 6×10^5 cells/6-cm dish, and 24 h later, 3.6 µg of pCAGGS vectors encoding one of the WT or mutant SHP-2 cDNAs and 0.9 µg of pcDNA3 containing HA-tagged extracellular signal-regulated kinase (ERK)2 cDNA were cotransfected using 8 µl of LipofectAMINE reagent and 12 µl of PLUS reagent. Two major mutants in NS, D61N and E76D, were used for this study. Three hours later, the medium was changed to DMEM containing 10% FCS. At 48 h after transfection, cells were unstimulated or stimulated with 20 ng/ml of epidermal growth factor (EGF) for 5, 30, 60, 90, and 120 min.

Immunoprecipitation of HA-ERK2 was performed, as described previously (Takeda et al. 1999). The 293 cells were washed twice with PBS, and immediately frozen in liquid nitrogen. The cells were lysed on ice in 0.3 ml of ice-cold RIPA buffer [20 mM Tris–HCl (pH 7.5), 140 mM NaCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 1% (v/v) NP-40 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 3 µl phosphatase inhibitor (Sigma)]. The lysates were centrifuged at 14,000 *g* for 15 min at 4°C. For immunoprecipitation, the resultant supernatants were incubated with anti-HA antibody (clone12CA5, gifted by Dr. Matozaki) for 2 h at 4°C and then incubated with 30 µl of protein G-Sepharose beads (Amersham) for 1 h on a shaker. The beads were washed twice with 800 µl wash buffer [20 mM Tris–HCl (pH 7.5), 140 mM NaCl, 0.1% (v/v) Triton X-100] and boiled for 5 min in 2× SDS sample buffer. Immunoprecipitants were subjected to SDS-polyacrylamide gel electrophoresis (10% gel), transferred to nitrocellulose membrane, and probed with the indicated antisera. All the membranes were visualized using enhanced chemi-

luminescence (ECL) Western blotting Detection System Kit (Amersham Pharmacia Biotech, Chalfont, UK).

Reporter assay

One day before the transfection, the 293 cells were plated in 6-well plates with a density of 5×10^5 cells per well. Cells were transiently transfected using LipofectAMINE plus reagent with 1 μg pSREluc, 0.1 μg of pRLTKluc, and 1 μg of SHP-2 wild or mutant expression constructs. Four mutants in the N-SH2 domain from NS patients were introduced in 293 cells. Eighteen hours after transfection, the cells were serum-starved in DMEM for 24 h. For EGF stimulation, cells were unstimulated or stimulated with 20 ng/ml of EGF for 5 h. Cells were harvested in passive lysis buffer, and luciferase activity was assayed using a dual luciferase assay kit (Promega, Madison, WI, USA). Renilla luciferase expressed from pRLTKluc was used to normalize the transfection efficiency. The experiments were performed in triplicate. Data are shown as mean ± SEM. Statistical analysis was performed with the Statview 4.0 package (AVACUS Corporation, Berkeley, CA, USA).

Results

Mutation analysis in patients with NS

Sequencing analysis in 41 affected individuals with NS revealed nine different missense mutations in 16 patients (15 sporadic cases and one index case of familial origin) (Table 1). Ten patients had mutations in the N-SH2 domain (Y63C in three patients; D61N, Q79R, and T73I in two patients each; and F71I in one patient). The novel F71I mutation identified in patient 6 was inherited from his father, who exhibited only ptosis. The F71I mutation was not detected in 83 controls (data not shown), suggesting the mutation was not likely to be a polymorphism. Although the D61N, Y63C, and Q79R mutations were detected in more than two patients, their clinical findings, including facial anomalies and heart defects, varied significantly. Patient 8, who had the T73I mutation, exhibited JMML at 2 months of age. Her leukocytosis persisted for 20 years after remission. Four mutations in the PTP domains were detected in six patients. Interestingly, patient 15 with the S502T mutation had an episode of transient abnormal myelopoiesis at 3 months of age and was associated with neuroblastoma at 6 months, suggesting the extending characteristics of the mutation (Kondoh et al. 2003). The analysis of her parents' DNA indicated the de novo mutation.

Phosphatase activities of mutants identified in NS

Structural analysis suggested that mutant proteins have an altered phosphatase activity. We transfected ten SHP-2 mutants, including nine mutants identified in NS patients in this study, and the E76A mutant as the positive control. Remarkable activation (six-fold to 12-fold compared with the activity of WT) was observed in the immune complexes derived from cells expressing the constructs carrying the D61N, F71I, or T73I mutation located in the N-SH2 domain (Fig.1). The activities of the D61N and F71I mutants were higher than that of the E76A mutant. Mild activation (two-fold to 3.9-fold) was observed in the immune complexes derived from cells transfected with constructs harboring the Y63C or Q79R mutation in the N-SH2 domain and constructs with the I282V, N308D, S502T, or M504V mutation in the PTP domain. Interestingly, all nine mutations had activated phosphatase activity.

PTPN11 mutation detection and phosphatase assay in childhood leukemia

In order to screen *PTPN11* mutation in leukemia cells, we sequenced exons 3 and 13 in the *PTPN11* gene from genomic DNA extracted from 29 leukemic children. Two mutations were detected in three cases (Fig.2). The A72V mutation was identified in bone marrow from one case with AML (M0 according to FAB classification) and in bone marrow from one patient with MDS (refractory anemia with excess blasts) (Monosomy 7). The novel G503V (1508G > T) mutation was detected in a bone-marrow-derived cell line from a JMML patient. The phosphatase activity in an immune complex precipitated from cells overexpressing the A72V mutant showed a 5.2-fold increase and that from cells over expressing the G503V mutant showed a 1.4-fold increase compared with the WT cDNA (Fig.2b).

In order to compare the phosphatase activity of mutations found in NS and leukemia at the same residue, we examined the phosphatase activity of two mutants occurring at D61 and E76. The activity of D61N (NS) was equivalent to that of D61Y (leukemia) (Fig.2c). In contrast, the activity of E76A (leukemia) was 4.1-fold that of E76D (NS) (Fig.2d).

Effects of mutants on MAPK pathway

Since SHP-2 has been known essentially as a positive regulator of the RAS/MAPK pathway (Feng 1999), we examined whether mutants activated ERK, a kind of MAPK, in the presence of EGF. In the absence and presence of EGF, the ERK activation was examined using an anti-phospho-ERK antibody that detected phosphorylated ERK. In cells expressing WT SHP-2 and ERK plasmids, the phosphorylation of ERK2 was maximum at 5 min after EGF treatment, and sustained activation persisted for 120 min (Fig.2a). In cells expressing the D61N or the E76D mutant, the maximum activation of ERK2 at 5 min was almost the same as in WT-transfected cells, but the activation decreased more