receptor in taste bud cells of mice (*12*). The mechanisms of this suppressive effect have been explained by the fact that leptin increases outward K^+ currents in taste bud cells *via* leptin receptors, leading to a reduction in excitability of the taste bud cells (*13*). The neural and behavioral sensitivities to sweet stimuli have been shown to be enhanced in db/db mice, an animal model for obesity, in the presence of a mutation of the *LEPR* (*11*).

Although it has been reported that genetic variations underlie specific eating patterns (*14*) and sweet preference is considered to be a risk factor for obesity, few reports have shown an association between them (*15*), and none have examined whether human *LEP* and *LEPR* polymorphisms have effects on sweet preference. However, there have been several reports indicating associations between *LEP/LEPR* polymorphisms and obesity (*5*). In the present study, we examined the associations of *LEP/LEPR* polymorphisms with sweet preference and attempted to determine whether these polymorphisms are associated with obesity because of their relationship to sweet preference.

Methods

Subjects

Between April 2002 and February 2004, we recruited 3,653 residents who had been participating in a public health physical checkup for citizens living in Suita City, located in the northern area of Osaka, Japan, since 1991. All subjects gave their written informed consent for the study, including genotyping. The study design was approved by the institutional review board and the ethics committee of the National Cardiovascular Center.

Measurements of Clinical Parameters

Blood pressure was determined after at least 10 min of rest in a sitting position. The final blood pressure value was the mean of 2 physician-obtained measurements (performed more than 3 min apart). Hypertension was defined as systolic blood pressure (SBP) ≥140 mmHg, diastolic blood pressure (DBP) ≥90 mmHg, or current use of antihypertensive agents. Diabetes mellitus (DM) was defined as fasting blood glucose ≥126 mg/dL, HbA1c ≥6.5%, or the current use of insulin or oral anti-diabetic agents. Hyperlipidemia was defined as total cholesterol ≥220 mg/dL, triglyceride ≥150 mg/dL, or the current use of anti-hyperlipidemia agents. Body mass index (BMI) was calculated by dividing weight (kg) by height (m) squared. For our analysis of obesity, we defined subjects in the top 25% of BMI results (above the third quartile) in each 5-year age group as obese, while those in the bottom 25% of BMI subjects were used as controls.

Semi-Quantification of Sweet Preference

A questionnaire designed to measure whether subjects had a "sweet tooth" was administered to each subject. The questionnaire consisted of a single question: "Do you like things that taste sweet?" to which the following responses were available: 1: No, I hate them; 2: No, I don't like them very much; 3: Neither yes or no; 4: Yes, I like them; and 5: Yes, I like them very much. The written answers were confirmed during an interview. To eliminate crossover, subjects who selected response number 5, above, were considered to have a sweet preference ($n=1,751$) and those that selected response numbers 1, 2, or 3 were defined as controls ($n=869$).

Polymorphism Study

Genomic DNA samples were collected from peripheral leukocytes using an NA-3000 (Kurabo Industries Ltd., Osaka, Japan) and initial polymorphism screening was performed using 24 DNA samples. All detected variations were further confirmed by a direct sequencing method using an ABI 3700 (Applied Biosystems, Foster City, USA). The genotypes were then determined using a TaqMan PCR system (ABI PRISM 7900HT; Applied Biosystems).

Statistical Analysis

The associations between sweet preference and obesity, the polymorphisms and sweet preference, and the polymorphisms and obesity were investigated using logistic regression analysis, with consideration of potential confounding factors (age, sex, smoking habit, drinking habit, and comorbidity of hypertension, diabetes, and hyperlipidemia). In addition, a Cochran-Armitage trend (CA) test was used to analyze the associations between the polymorphisms and sweet preference. Further, an analysis of covariance was used together with a path analysis, which is the statistical method commonly used to evaluate the relative importance of various causes (*16*), to investigate whether the polymorphisms were related to obesity based on their relationship to sweet preference. All analyses were performed using Statview software (SAS Institute Inc., Cary, USA). Values of $p<0.05$ were considered to be statistically significant.

Results

LEP/LEPR Polymorphisms

Table 1 shows the characteristics and frequencies of the *LEP/LEPR* polymorphisms in 24 control subjects, along with the linkage disequilibrium (LD) patterns. Since the results indicated that *LEPR* N656K and rs3790439 had strong LD values, we further investigated rs3790439 instead of N656K. To examine the significant difference between the expected results and the raw ones in the genotype distribution, we used

Table 1. Characters of *LEP* and *LEPR* Polymorphisms

A: Locations of *LEP* and *LEPR* polymorphisms

SNPs	db SNP ID	Region	Flanking sequence
LEP			
G−2548A	rs7799039	promoter	acagggttgc[g/a]ctgatcctcc
A19G	rs2167270	5′-UTR	cgcagcgcca[a/g]cggttgcaag
LEPR			
R109K	rs1137100	exon 4	attgaaggaa[g/a]gacattgtt
R223Q	rs1137101	exon 6	gtaattttcc[g/a]gtcacctcta
N656K	rs8179183	exon 14	ctatgaaaaa[c/g]gagaaaaatg
rs3790439	rs3790439	intron 16	tcatgactag[g/a]taattagaag

B: Evaluation of patterns of linkage disequilibrium in *LEP* and *LEPR* polymorphisms

D'	r^2	
	G−2548A	A19G
LEP		
G−2548A		0.745
A19G	0.994	

D'	r^2			
	R109K	R223Q	N656K	rs3790439
LEPR				
R109K		0.636	0.206	0.206
R223Q	1.00		0.390	0.390
N656K	0.503	0.707		1.00
rs3790439	0.503	0.707	1.00	

C: Frequency of *LEP* and *LEPR* polymorphisms

	Allele 1 homo (*n*)	Hetero (*n*)	Allele 2 homo (*n*)
LEP			
G−2548A	GG 4.9% (179)	GA 34.2% (1,246)	AA 60.9% (2,222)
A19G	AA 3.0% (109)	AG 29.2% (1,065)	GG 67.9% (2,479)
LEPR			
R109K	GG 61.7% (2,243)	GA 33.8% (1,231)	AA 4.5% (163)
R223Q	GG 72.8% (2,646)	GA 24.9% (907)	AA 2.3% (83)
rs3790439	GG 1.0% (37)	GA 19.2% (701)	AA 79.8% (2,913)

SNP, single nucleotide polymorphism; *LEP*, leptin gene; *LEPR*, leptin receptor gene.

Hardy-Weinberg equilibrium. There were no significant differences in genotype frequencies of the polymorphisms between males and females (data not shown), which were nearly identical to the results in an Asian population reported by the National Center of Biotechnology Information (NCBI). Based on these results, we selected a total of 5 polymorphisms; G−2548A and A19G for *LEP*, and R109K, R223Q, and rs3790439 for *LEPR*.

Sweet Preference and Clinical Characteristics

The distribution of answers to the sweet preference question in the questionnaire was not a normal probability distribution, and response number 5 (like sweet-tasting things very much) was chosen with the highest frequency (response 1: 5.9%; 2: 13.8%; 3: 11.6%; 4: 5.9%; 5: 62.9%). Thus we used not covariance analysis but logistic regression analysis. There was a significant difference between males and females in the choice of response number 5, as females more frequently stated their preference for a sweet taste (males: 58.6%; females: 66.6%).

The clinical characteristics of the study subjects are shown in Table 2. When clinical characteristics were compared between the subjects with a sweet preference and the controls,

Table 2. Clinical Demography

Variables	A: Sweet tooth and control			B: With *LEPR* 109KK genotype and others			C: With *LEP*19GG genotype and others		
	Sweet tooth (n=1,751)	Control (n=869)	p value	*LEPR* 109KK (n=163)	Others (n=3,474)	p value	*LEP* 19GG (n=2,479)	Others (n=1,174)	p value
Age (years old)	64.1±0.3	64.8±0.4	0.14	65.2±0.9	64.7±0.2	0.59	64.2±0.2	65.7±0.3	0.0002*
BMI (kg/m^2)	22.9±0.1	22.8±0.1	0.19	23.3±0.2	22.8±0.1	0.029*	22.9±0.1	22.7±0.1	0.29
SBP (mmHg)	128.1±0.5	130.5±0.6	0.023*	130.7±1.5	129.4±0.3	0.40	129.4±0.4	129.5±0.6	0.89
DBP (mmHg)	77.2±0.2	78.6±0.3	0.023*	78.0±0.7	77.8±0.2	0.79	77.9±0.2	77.5±0.3	0.33
T-cho (mg/dL)	209.5±0.9	206.0±1.2	0.38	207.4±2.7	208.0±0.6	0.83	208.1±0.7	207.6±1.0	0.66
TG (mg/dL)	103.3±1.6	109.7±2.7	0.16	105.0±5.1	106.2±1.2	0.83	106.5±1.5	105.3±2.0	0.66
LDL cholesterol (mg/dL)	128.4±0.8	123.0±1.1	0.016*	126.5±2.6	126.3±0.5	0.94	126.2±0.6	126.3±0.9	0.96
HDL cholesterol (mg/dL)	60.5±0.4	61.3±0.6	0.070	59.7±1.3	60.2±0.3	0.71	60.3±0.3	59.9±0.4	0.47
FBS (mg/dL)	95.7±0.4	97.0±0.6	0.45	96.0±1.2	96.0±0.3	0.99	96.1±0.3	95.9±0.4	0.81
HbA1c	5.35±0.01	5.39±0.02	0.19	5.34±0.04	5.37±0.01	0.54	5.37±0.01	5.36±0.01	0.92
f-Insulin (mg/dL)	5.7±0.1	5.6±0.2	0.41	5.5±0.3	5.5±0.1	0.98	5.6±0.1	5.3±0.1	0.051
HOMA-IR	1.38±0.03	1.42±0.07	0.54	1.36±0.11	1.35±0.02	0.92	1.38±0.03	1.28±0.03	0.039*
Hypertension (%)	29.2	32.9	0.055	31.3	31.1	0.97	31.2	31.3	0.92
Hyperlipidemia (%)	29.8	25.7	0.027*	23.9	29.3	0.14	28.2	30.8	0.10
Diabetes mellitus (%)	8.2	9.0	0.48	11.0	8.5	0.26	8.8	8.3	0.68
Current smoker (%)	14.4	21.3	<0.0001*	12.3	17.4	0.09	17.9	15.8	0.11
Alcohol consumer (%)	37.9	59.5	<0.0001*	46.0	45.8	0.96	47.0	43.5	0.052

LEP, leptin gene; *LEPR*, leptin receptor gene; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T-chol, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBS, fetal bovine serum; f-Insulin, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance. *Significant p values.

Table 3. Association of Sweet Preference with Obesity

	Obesity (+) (% (n))	Obesity (−) (% (n))	Odds ratio (95% CI)	p value
Total				
Sweet tooth	53.1 (483)	46.2 (195)	1.36 (1.06–1.75)	0.017*
Control	46.9 (427)	53.8 (227)		
Male				
Sweet tooth	54.9 (208)	45.1 (171)	1.57 (1.09–2.26)	0.016*
Control	43.5 (101)	56.5 (131)		
Female				
Sweet tooth	51.8 (275)	48.2 (256)	1.16 (0.82–1.65)	0.39
Control	49.5 (94)	50.5 (96)		

CI, confidence interval. Potential confounding factors were age, sex, smoking habit, drinking habit, and prevalence of hypertension, diabetes, and hyperlipidemia. *Significant p values.

the results indicated that low-density lipoprotein (LDL) cholesterol values were significantly higher in the subjects with a sweet preference and that SBP and DBP were lower in those groups (p values: LDL cholesterol, 0.016; SBP, 0.023; DBP, 0.023) (Table 2A). In addition, the ratios for smoking and alcohol habits were significantly lower (p values: current smoker, <0.0001; alcohol drinker, <0.0001) and the ratio for hyperlipidemia was higher (p values, 0.027) in those with a sweet preference. Also, Table 2B and C show the clinical characteristics of the subjects with and without the specific genotype *LEPR* 109KK or *LEP* 19GG.

With regard to the potentially confounding factors of age, sex, smoking habit, drinking habit, and comorbidity of hypertension, diabetes or hyperlipidemia, the subjects with a sweet preference tended toward obesity (odds ratio [OR], 1.36; 95% confidence interval [95% CI], 1.06–1.75; p value, 0.017) and the tendency was stronger in males than in females (p value, 0.016) (Table 3).

Table 4. Association of *LEP* and *LEPR* Polymorphisms with Sweet Preference

A: Association of *LEP* polymorphisms with sweet preference

LEP	Total		Male		Female	
	Sweet tooth	Control	Sweet tooth	Control	Sweet tooth	Control
G−2548A						
GG	79 (4.5)	42 (4.8)	41 (5.4)	24 (5.2)	38 (3.8)	18 (4.4)
GA	599 (34.3)	307 (35.4)	248 (32.8)	160 (34.7)	351 (35.5)	147 (36.2)
AA	1,069 (61.2)	518 (59.7)	468 (61.8)	277 (60.1)	601 (60.7)	241 (59.4)
OR (95% CI)	0.91 (0.77–1.09)		0.87 (0.68–1.11)		0.83 (0.47–1.49)	
p value	0.30 (dom)		0.26 (dom)		0.54 (rec)	
A19G						
AA	45 (2.6)	26 (3.0)	22 (3.0)	14 (2.9)	23 (2.3)	12 (2.9)
AG	499 (28.5)	273 (31.5)	216 (31.9)	147 (28.5)	283 (28.6)	126 (31.0)
GG	1,206 (68.9)	569 (65.6)	521 (65.1)	300 (68.6)	685 (69.1)	269 (66.1)
OR (95% CI)	0.82 (0.69–0.98)		0.78 (0.60–1.01)		0.87 (0.70–1.09)	
p value	0.032* (dom)		0.056 (dom)		0.23 (add)	

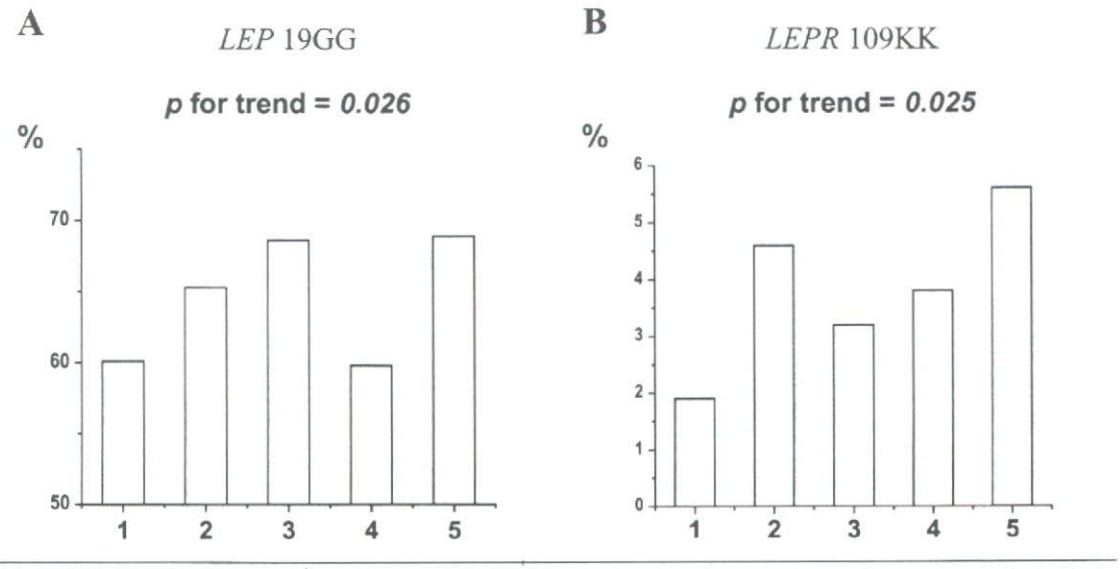
B: Association of *LEPR* polymorphisms with sweet preference

LEPR	Total		Male		Female	
	Sweet tooth	Control	Sweet tooth	Control	Sweet tooth	Control
R109K						
GG	1,075 (61.6)	553 (63.9)	468 (62.1)	302 (65.7)	607 (61.3)	251 (61.8)
GA	576 (33.0)	283 (32.7)	246 (32.6)	140 (30.4)	330 (33.3)	143 (35.2)
AA	93 (5.3)	30 (3.5)	40 (5.3)	18 (3.9)	53 (5.4)	12 (3.0)
OR (95% CI)	0.64 (0.42–0.98)		0.83 (0.67–1.02)		0.59 (0.31–1.13)	
p value	0.042* (dom)		0.081 (add)		0.11 (dom)	
R223Q						
GG	1,270 (72.9)	639 (73.9)	548 (72.5)	346 (75.2)	722 (73.2)	293 (72.3)
GA	429 (24.6)	211 (24.4)	189 (25.0)	104 (22.6)	240 (24.3)	107 (26.4)
AA	44 (2.5)	15 (1.7)	19 (2.5)	10 (2.2)	25 (2.5)	5 (1.2)
OR (95% CI)	0.66 (0.36–1.22)		0.84 (0.66–1.07)		0.54 (0.20–1.42)	
p value	0.18 (dom)		0.17 (add)		0.21 (dom)	
rs3790439						
AA	19 (1.1)	8 (0.9)	8 (1.1)	6 (1.3)	11 (1.1)	2 (0.5)
AG	332 (19.0)	157 (18.1)	138 (18.2)	73 (15.8)	194 (19.6)	84 (20.6)
GG	1,397 (79.9)	703 (79.9)	611 (80.7)	382 (82.9)	786 (79.3)	321 (78.9)
OR (95% CI)	1.37 (0.58–3.20)		1.16 (0.85–1.58)		2.13 (0.47–9.71)	
p value	0.47 (rec)		0.36 (dom)		0.33 (rec)	

LEP, leptin gene; *LEPR*, leptin receptor gene; OR, odds ratio; CI, confidence interval; add, additive; dom, dominant; rec, recessive. *Significant *p* values.

Associations of *LEP* and *LEPR* Polymorphisms with Sweet Preference and Obesity

The associations of *LEP* and *LEPR* polymorphisms with sweet preference are depicted in Table 4A and B, respectively. The results indicated that there was a significant association between the *LEP* A19G and *LEPR* R109K polymorphisms and sweet preference (*LEP* A19G, $p=0.032$ [dominant model]; *LEPR* R109K, $p=0.042$ [dominant model]). We also examined the associations by χ^2 tests, and the results indicated the same tendency (data not shown). Thus, subjects with *LEP* 19GG or *LEPR* 109KK tended to be subjects with a sweet preference. However, no correlation between *LEP* A19G and *LEPR* R109K was observed. In the Cochran-Armitage trend (CA) test, associations between sweet preference and *LEP* A19G and *LEPR* R109K were shown much more clearly (Fig. 1). Those results indicated that there was a significant association between sweet preference and *LEP* 19GG (trend *p* value =0.026) and *LEPR* 109KK (trend *p* value =0.025). Further, the ratios of *LEP*



	1	2	3	4	5	Total
Total (*n*)	163	383	322	164	1,750	2,782
***LEP* 19GG**	98	250	221	98	1,206	1,873
Ratio (%)	60.1	65.3	68.6	59.8	68.9	67.3

	1	2	3	4	5	Total
Total (*n*)	159	366	311	157	1,651	2,644
***LEPR* 109KK**	3	17	10	6	93	129
Ratio (%)	1.9	4.6	3.2	3.8	5.6	4.9

Fig. 1. *Distribution of answers to the sweet preference question by subjects with the LEP 19GG (A) and LEPR 109KK (B) polymorphisms. The ratios of LEP 19GG and LEPR 109KK occurrence gradually increased with increases in the degree of sweet preference, and there was a significant association between sweet preference and LEP 19GG (trend p value = 0.026) and LEPR 109KK (trend p value = 0.025).*

19GG and *LEPR* 109KK occurrence gradually increased with higher levels of sweet preference.

Next, we investigated the association of *LEP* and *LEPR* polymorphisms with serum leptin levels, since some reports have noted that the *LEP* G−2548A and *LEP* A19G polymorphisms are associated with serum leptin levels. (*10*, *17–19*) We indeed found that both *LEP* G−2548A (total, male, and female) and *LEP* A19G (total and male) were significantly associated with serum leptin levels in our subjects (data not shown). However, we concluded that *LEP* A19G does not affect sweet preference through its change of serum leptin levels, since no correlation was found between sweet preference and serum leptin level (data not shown). We also studied the association of these polymorphisms with obesity (Table 5), and the results indicated that there was a strong association between the *LEPR* polymorphisms (R109K and R223Q) and obesity (*LEPR* R109K, $p=0.0076$ [dominant]; *LEPR* R223Q, $p=0.025$ [dominant]). We also evaluated the mean value of BMI among each genotype subject, and the results showed the same tendency (data not shown). Subjects with *LEPR* 109KK or 223QQ tended toward obesity and the tendency with *LEPR* 109KK was stronger in males.

Based on our results, *LEPR* 109KK was considered to be associated with both sweet preference and obesity. Then, we investigated whether *LEPR* 109KK had an effect on obesity by means of its relationship to sweet preference. First we confirmed that there were no interactions between sweet preference and the genotypes affecting obesity. We further utilized a path analysis to consider several potential confounding factors—age, sex, smoking habit, drinking habit, and comorbidity of hypertension, diabetes or hyperlipidemia—and we used obesity as an induced variable, and both *LEPR* 109KK presence and sweet preference as objective variables. We found that there was a significant association of both *LEPR* 109KK and sweet preference with obesity, and that *LEPR* 109KK had an effect on obesity *via* its induction of sweet preference (Fig. 2). The contribution of sweet preference to obesity was calculated to be about 2.8%, so we speculated that *LEPR* 109KK might also affect obesity through other means.

Discussion

In the present study, sweet preference was shown to be associated with obesity, and the *LEP* A19G and *LEPR* R109K polymorphisms were significantly associated with sweet preference. It was also revealed that *LEPR* 109KK had an effect on obesity *via* its induction of sweet preference. *LEPR* 223QQ was also shown to be associated with obesity but its

Table 5. Association of *LEP* and *LEPR* Polymorphisms with Obesity

A: Association of *LEP* polymorphisms with obesity

LEP	Total		Male		Female	
	Obesity	Control	Obesity	Control	Obesity	Control
G−2548A						
GG	43 (13.9)	42 (12.1)	27 (13.9)	20 (12.1)	16 (3.3)	22 (4.5)
GA	300 (46.5)	305 (42.7)	133 (47.9)	147 (40.9)	167 (34.3)	158 (32.6)
AA	570 (39.6)	562 (45.2)	266 (38.1)	258 (47.0)	304 (62.4)	304 (62.8)
OR (95% CI)	1.10 (0.70–1.75)		1.55 (0.82–2.93)		0.72 (0.36–1.44)	
p value	0.68 (rec)		0.17 (rec)		0.35 (rec)	
A19G						
AA	30 (3.3)	26 (2.9)	15 (3.5)	14 (3.3)	15 (3.1)	12 (2.5)
AG	239 (26.2)	265 (29.1)	111 (26.1)	125 (29.3)	128 (26.3)	140 (28.8)
GG	644 (70.5)	621 (68.1)	300 (70.4)	287 (67.4)	344 (70.6)	334 (68.7)
OR (95% CI)	0.89 (0.72–1.10)		0.88 (0.64–1.20)		0.89 (0.67–1.18)	
p value	0.27 (dom)		0.41 (dom)		0.40 (dom)	

B: Association of *LEPR* polymorphisms with obesity

LEPR	Total		Male		Female	
	Obesity	Control	Obesity	Control	Obesity	Control
R109K						
GG	559 (61.4)	571 (62.9)	269 (63.6)	274 (64.6)	290 (59.4)	297 (61.4)
GA	301 (33.0)	309 (34.0)	129 (30.5)	141 (33.3)	172 (35.2)	168 (34.7)
AA	51 (5.6)	28 (3.1)	25 (5.9)	9 (2.1)	26 (5.3)	19 (3.9)
OR (95% CI)	0.51 (0.32–0.84)		0.28 (1.13–0.63)		0.73 (0.39–1.37)	
p value	0.0076* (dom)		0.0022* (dom)		0.33 (dom)	
R223Q						
GG	684 (74.9)	664 (73.1)	322 (75.8)	321 (75.4)	362 (74.2)	343 (71.2)
GA	201 (22.0)	229 (25.2)	92 (21.6)	98 (23.0)	109 (22.3)	131 (27.2)
AA	28 (3.1)	15 (1.7)	11 (2.6)	7 (1.6)	17 (3.5)	8 (1.7)
OR (95% CI)	0.47 (0.24–0.91)		0.46 (0.17–1.27)		0.45 (0.18–1.09)	
p value	0.025* (dom)		0.13 (dom)		0.077 (dom)	
rs3790439						
AA	8 (0.9)	9 (1.0)	4 (0.9)	4 (0.9)	4 (0.8)	5 (1.0)
AG	170 (18.6)	189 (20.7)	71 (16.7)	79 (18.5)	99 (20.3)	110 (22.6)
GG	736 (80.5)	714 (78.3)	351 (82.4)	343 (80.5)	385 (78.9)	371 (76.3)
OR (95% CI)	0.84 (0.66–1.07)		1.34 (0.30–6.05)		0.79 (0.57–1.08)	
p value	0.15 (dom)		0.70 (rec)		0.13 (dom)	

LEP, leptin gene; *LEPR*, leptin receptor gene; OR, odds ratio; CI, confidence interval; dom, dominant; rec, recessive. *Significant *p* values.

association was found to be weaker than that of *LEPR* 109KK.

It has been reported that sweeteners activate transduction cascades in sweet-responsive cells, with one involving cAMP and the other involving inositol trisphosphate (IP_3) in taste bud cells (*20*). Sugar increases intracellular cAMP, which activates protein kinase A (PKA) and closes K^+ channels mediated by phosphorylation, which in turn leads to influx of Ca^{2+} through voltage-gated Ca^{2+} channels. In contrast, artificial sweeteners such as saccharin elevate IP_3 levels, which induces Ca^{2+} release from intracellular stores and an elevation of intracellular Ca^{2+} concentration. The IP_3 pathway is proposed to result in closure of K^+ channels *via* protein kinase C (PKC)–mediated phosphorylation activated by concomitantly produced diacylglycerol (DAG) (*21*). Therefore, both second messenger pathways may converge on the same K^+ channels in taste bud cells. The recent studies (*11*, *13*) noted that leptin increased the K^+ conductance of taste cells, leading to hyperpolarization and reduction of cell excitability, and leptin suppressed behavioral responses to sweet substances. Thus we

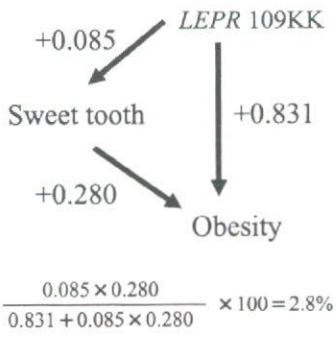


Fig. 2. *Correlations among LEPR 109KK, sweet preference, and obesity. Each value shown represents the coefficient of covariance analysis. There was a significant association of both LEPR 109KK and sweet preference with obesity, and LEPR 109KK had an affect on obesity via its induction of sweet preference. The contribution of sweet preference to obesity was calculated to be about 2.8%.*

considered that the *LEPR* R109K or the *LEP* A19G polymorphism might affect the molecular mechanisms of leptin activation of K^+ currents like the currents in pancreatic β cells (*22*) and hypothalamic neurons (*23*).

The *LEPR* R109K polymorphism is located in exon 4 of the gene, and changes an arginine to a lysine. *LEPR* R109K is not located in the intracellular domain, which is essential for the JAK-STAT signal transduction pathway (*24*, *25*) of this peptide, nor in the leptin-binding site (*26*). The functional relevance of the polymorphism has not been clarified, though previous reports have found that it is associated with obesity, including in Asians (*8*). Thus, our results may provide useful information for the treatment and prevention of obesity, while they also suggest a new biological pathway for the role of *LEPR* R109K in sweet preference, which might induce obesity. In addition, our findings may inform future clinical attempts to apply genetic information to the treatment of obesity, though additional investigations are needed.

Another intriguing polymorphism, *LEP* A19G, is located in the 5′ untranslated region (5′-UTR) of the *LEP* gene. It was previously reported that obese individuals with the A19 allele had significantly higher leptin concentrations as compared to subjects with 19GG (*19*), which is supported by our results. Although both the *LEP* G−2548A and A19G polymorphisms are associated with serum leptin levels, only A19G had a significant association with sweet preference. Since the rs1349419 polymorphism in the 5′-flanking region of *LEP* is considered to modify transcription factor–binding sites (*13*, *24*), it is intriguing that A19G showed a strong LD with rs1349419. Thus, it could be possible that A19G has an effect on sweet preference through functional changes of the rs1349419 polymorphism. In addition, the *LEPR* R109K polymorphism might also influence conformational changes of *LEPR*, thus affecting sweet preference, as it has been reported that the leptin receptor exists as a multimeric complex and leptin activates the receptor by inducing a conformational change (*26*).

In clinical practice, obesity is closely associated with increased morbidity and mortality caused by cardiovascular diseases, diabetes, and hypertension (*1*, *27–30*). Therefore, obese patients should be given dietary instruction, though many with obesity or lifestyle-related diseases are dissatisfied when sweets are restricted. Our results indicate that individuals with the *LEPR* 109KK allele tend to prefer sweets naturally, and therefore dietary therapy for those individuals may have to be well thought out. We consider that our data regarding the *LEPR* R109K polymorphism could lead to its use as a marker for personalized medical intervention when treating obesity and lifestyle-related diseases. Additionally, in this study, there was a gender difference in the distribution of answers in regard to sweet preference and the association between the polymorphisms and sweet preference. However, it was difficult to accurately determine the reason for this gender difference, because sweet preference is also influenced by various unquantifiable factors, such as cultures and customs.

In conclusion, we found associations among sweet preference, obesity and *LEPR* polymorphisms. Further, the role of *LEPR* R109K in sweet preference in the present subjects was found to be a possible cause of obesity. This is the first report showing a gene for sweet preference in humans, and we consider that our findings will contribute to better treatment of obesity and lifestyle-related diseases.

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Pioglitazone treatment stimulates circulating CD34-positive cells in type 2 diabetes patients

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ABSTRACT

Circulating bone marrow derived immature cells, including CD34-positive ($CD34^+$) cells, contribute to maintenance of the vasculature, not only as a pool of endothelial progenitor cells (EPCs), but also as a source of growth/angiogenesis factor. We hypothesized that the thiazolidineone compound pioglitazone could stimulate the circulating $CD34^+$ cells in diabetic patients. Thirty-four patients with type 2 diabetes received 15–30 mg pioglitazone for 24 weeks. The number of circulating $CD34^+$ cells significantly increased at 12 and continued this effect for 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 cells/μl at 0, 12 and 24 weeks, respectively). The change of $CD34^+$ cell levels ($\Delta CD34^+$ cells) between 0 and 12 weeks was significantly correlated with the change of high sensitive C reactive protein levels (Δhs-CRP) and change in adiponectin levels (Δadiponectin) ($r = -0.412$, $r = 0.359$, respectively). Our study demonstrated that pioglitazone treatment increased circulating $CD34^+$ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

1. Introduction

Endothelial dysfunction plays a pivotal role in the progression of the atherosclerosis. Circulating EPCs contribute to the maintenance of vascular homeostasis and repair. They also play an important role in the maintenance of vascular endothelial function [1,2]. In diabetic patients, both a decrease in number and function of circulating EPCs are reported, suggesting that circulating EPCs participate in diabetic vascular complications [3].

Recent studies have identified circulating bone marrow derived immature cells, including $CD34^+$ cells, contribute to maintenance of the vasculature, not only as a pool of EPCs, but also as a source of growth/angiogenesis factor [4]. In fact, one recent report indicates that circulating $CD34^+$ cells are more strongly correlated with cardiovascular risk than circulating $CD34^+$/kinase insert domain receptor $(KDR)^+$ cells generally regarded as EPCs [5]. We have also reported that circulating $CD34^+$ cell levels are associated with cerebral infarction [6]. These findings indicate that persistent stimulation of $CD34^+$ cells may be a useful method to repair endothelial injury and microcirculation, and to suppress the progression of atherosclerotic disease at least theoretically. Recent experimental and clinical studies demonstrate that thiazolidinediones, peroxisome-proliferator-activated receptor γ (PPARγ) agonists, has the effects on the prevention of atherosclerosis including the maintenance of vascular endothelial function [7–9]. Therefore, we hypothesized that the thiazolidineone

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compound pioglitazone could stimulate the circulating $CD34^+$ cells in diabetic patients.

2. Methods

2.1. Study subjects

All subjects gave a written informed consent. The study was approved by the local ethics committee. Thirty-four patients with type 2 diabetes (age 60 ± 10, M/F; 18/16, HbA1c 9.3 ± 1.4%) received 15 or 30 mg pioglitazone for 24 weeks (15 mg; 31 patients, 30 mg; 3 patients). Other medications for diabetes, hypertension and hyperlipidemia were unchanged throughout the study. Insulin was given to 9 patients. Sulfonylurea was given to 15 patients. Biguanide was given to 21 patients. Alpha glucosidase inhibitor was given to 10 patients. Angiotensin converting enzyme inhibitor and/or angiotensin receptor blocker was given to 21 patients. Statin was given to 18 patients. Sixteen patients afflicted with cardiovascular diseases (CVD). Eighteen patients afflicted with nephropathy, 14 patients afflicted with retinopathy, and 15 patients afflicted with neuropathy.

2.2. Measurement of $CD34^+$ cells

Three milliliters of heparinized peripheral blood were obtained after 12-h fasting and measured $CD34^+$ cells. The precise number of circulating $CD34^+$ cells was quantified as we described previously [10]. We evaluated circulating $CD34^+$ cells with Stem-Kit™ (BeckmanCoulter, Marseille, France) according to manufacturers' protocols. These protocols are based on International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines [11], and are frequently used for quantification of $CD34^+$ cells mobilized into peripheral blood. To increase the reproducibility of $CD34^+$ cell counts, the protocol of Stem-Kit was modified as follows: the blood sample volume, antibodies and lysing solution were doubled. After adding 30 μl of internal control (Stem count: BeckmanCoulter), samples were centrifuged for 5 min at 450 × *g* and 3860 μl of supernatant was removed carefully with a pipet. Samples were analyzed by Coulter CYTOMICS™ FC500 & XL-system II software (BeckmanCoulter) for 6 min each.

2.3. Other laboratory analysis

Blood samples were taken after 12-h fasting to measure adiponectin and, high sensitive C-reactive protein (hs-CRP) concentrations. Serum adiponectin and concentration was measured by enzyme-linked immunosorbent assay (SRL, Tokyo, Japan). Serum hs-CRP concentration was measured by latex nephelometry method (SRL, Tokyo, Japan). We also measured HbA1c, total cholesterol, HDL cholesterol and triglyceride levels.

2.4. Statistical analysis

Data was expressed using the mean ± S.D. The Student's t-test was used to compare parameter changes over time. The strength of correlation between variables was performed using Spearmann's correlation coefficient.

3. Results

3.1. Effects of pioglitazone on glucose and lipid metabolism

Treatment of pioglitazone significantly decreased HBA1c levels (9.3 ± 1.4, 7.4 ± 1.2 and 7.5 ± 1.7% at 0, 12 and 24 weeks, respectively). Systemic blood pressure levels did not change throughout the study period. BMI did not change throughout the study period (26.8 ± 3.2, 27.5 ± 3.0 and 27.9 ± 3.3 at 0, 12 and 24 weeks, respectively). Total cholesterol and triglyceride levels did not change throughout the study, whereas HDL cholesterol levels significantly increased at 12 and 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 mmol/l at 0, 12 and 24 weeks, respectively).

3.2. Effects of pioglitazone on adiponectin and inflammatory marker

The inflammatory marker, hs-CRP significantly decreased at 12 and 24 weeks (1518 ± 2350, 840 ± 975, and 838 ± 904 ng/ml at 0, 12, and 24 weeks, respectively). Serum adiponectin levels significantly increased at 12 and 24 weeks (5.0 ± 2.2, 13.5 ± 6.7 and 13.8 ± 8.4 μg/ml at 0, 12 and 24 weeks, respectively). The change in adiponectin levels between 0 and 12 weeks (Δadiponectin) of 30 mg pioglitazone was significantly larger than 15 mg of pioglitazone (15 mg; 7.9 ± 4.7 vs. 30 mg; 19.6 ± 2.5, $p < 0.05$), whereas there was no significant difference in the change in hs-CRP levels (Δhs-CRP) between 15 mg and 30 mg of pioglitazone (15 mg; 267 ± 322 vs. 30 mg; 480 ± 1883).

3.3. Effects of pioglitazone on circulating $CD34^+$ cell level

The number of circulating $CD34^+$ cells significantly increased at 12 and 24 weeks (0.90 ± 0.48, 1.10 ± 0.50, and 1.10 ± 0.57 cells/μl at 0, 12, and 24 weeks, respectively (Fig. 1). This effect was found in both patients with CVD and without CVD (patients with CVD; 0.81 ± 0.51, 1.05 ± 0.46 and 1.04 ± 0.50 cells/μl at 0, 12 and 24 weeks, respectively, $n = 16$, patients without CVD; 0.98 ± 0.41,

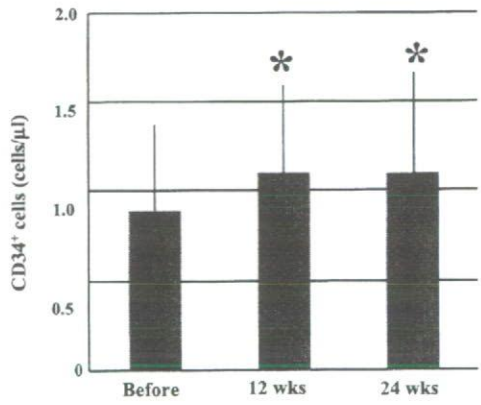

Fig. 1 – CD34+ cell level at 0, 12 and 24 weeks, *$p < 0.05$ vs. 0 week.

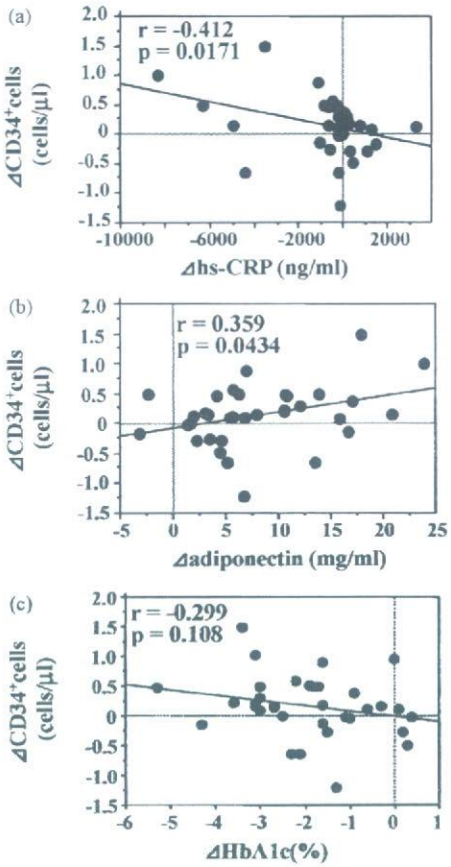

Fig. 2 – Correlation between ΔCD34⁺ cells and ΔhsCRP ($r = -0.412$, $p = 0.017$) (a), correlation between ΔCD34⁺ cells and Δadiponectin ($r = 0.359$, $p = 0.043$) (b), and correlation between ΔCD34⁺ cells and ΔHbA1c ($r = -0.299$, $p = 0.108$) (c).

1.15 ± 0.57 and 1.15 ± 0.65 cells/μl at 0, 12 and 24 weeks, respectively, $n = 18$). There was no significant difference in the change in CD34⁺ cell level (ΔCD34⁺ cells) between 15 mg and 30 mg of pioglitazone (15 mg; 0.07 ± 1.01 vs. 30 mg; 0.14 ± 0.32).

3.4. *Factors involved in the stimulation of CD34⁺ cells*

We next investigated which factors were correlated with the stimulation of CD34⁺ cells. ΔCD34⁺ cells were significantly correlated with Δhs-CRP in univariate analysis ($r = -0.412$, $p = 0.017$) (Fig. 2a). Further, Δadiponectin correlated with Δ CD34⁺ cells ($r = 0.359$, $p = 0.043$) (Fig. 2b). On the other hand, change in HbA1c levels (ΔHbA1c) ($r = -0.299$, $p = 0.108$) (Fig. 2c), change in HDL-C levels (ΔHDL-C) ($r = 0.253$, $p = 0.168$) and change in triglyceride levels (Δtriglycerides) ($r = 0.0072$, $p = 0.969$), were not significantly correlated to Δ CD34⁺ cells.

4. Discussion

Accumulating evidence shows that PPARγ agonists have anti-atherosclerotic actions other than their blood glucose level reduction effects [7,9]. One recent report showed that pioglitazone treatment could stimulate circulating EPCs in patients with coronary artery disease and normal glucose tolerance [12]. In this study, we demonstrated that pioglitazone treatment also increased circulating CD34⁺ cells and this effect continued for 24 weeks in type 2 diabetic patients. We studied the effects of pioglitazone on the stimulation of CD34⁺ cells but not CD34⁺/KDR⁺ cells regarded as EPCs. However, these circulating CD34⁺ cells have the capacity to participate in neovascularization of ischemic tissue. Indeed, their administration enhances the repair of ischemic tissue in ischemic stroke model [13] and improves myocardial circulation in myocardial infarction model [14]. Clinically, circulating CD34⁺ cell levels were reported to be correlated with cerebral blood flow in hypoperfusion area [6] and formation of collateral vessels in stroke patients [15]. These reports suggest that CD34⁺ cells may play a role in the maintenance of microcirculation. One recent clinical trial, PROactive Study, demonstrated that pioglitazone treatment could prevent cardiovascular events including stroke in type 2 diabetic patients [16]. Taken together, it is suggested that the stimulation of CD34⁺ cells may partly contribute to the preventive effects of pioglitazone on cardiovascular diseases. Our study also demonstrated that pioglitazone treatment increased circulating CD34⁺ cells in type 2 diabetic patients irrespective of with or without CVD, suggesting that pioglitazone treatment may be useful for primary prevention as well as secondary prevention of diabetic macroangiopathy.

It has been reported that the number of circulating EPCs is inversely correlated with HbA1c levels [3]. Since pioglitazone treatment significantly decreased HbA1c levels and this study did not have control group, we could not exclude the possibility that the stimulation of CD34⁺ cells was associated with the improvement of glycemic control. However, the results of this study suggest that pioglitazone may be capable of stimulating circulating CD34⁺ cells independently of glycemic control because ΔCD34⁺ cells was not positively correlated with ΔHbA1c at levels that achieved statistical significance.

Adipocyte derived factors and inflammation participate in atherogenesis of type 2 diabetic patients. Accumulating evidence show that adiponectin, one of adipocyte derived factors, has anti-atherogenic properties, and hypoadionectinemia was reported to be associated with endothelial dysfunction [17]. Pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in metabolic syndrome subjects [8], suggesting that these effects contribute to the anti-atherosclerotic action of pioglitazone. In this study, we also demonstrated that pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in type 2 diabetes patients. Interestingly, ΔCD34⁺ cells were significantly correlated with Δhs-CRP and Δadiponectin. An in vitro study showed that CRP impaired EPC migration and function [18]. In clinical study, it has been reported that circulating EPCs were inversely correlated to serum interleukin 6 levels [19]. These reports suggested that chronic inflammation may be involved in the regulation of EPCs. One recent clinical study showed that circulating EPCs were positively correlated to serum adiponectin levels in patients with coronary artery disease [20]. Another report showed that

adiponectin treatment increased EPC number and migration [12]. Taken together, it is suggested that the inhibitory effects on chronic inflammation and the effect on adiponectin regulation of pioglitazone may be directly or indirectly involved in the increase of $CD34^+$ cells. However, further study is necessary to delineate this hypothesis.

In conclusion, our study demonstrated that pioglitazone treatment increased circulating $CD34^+$ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

Conflict of interest

There are no conflicts of interest.

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Mutation Site Dependent Variability of Cardiac Events in Japanese LQT2 Form of Congenital Long-QT Syndrome

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Background In the LQT2 form of long QT syndrome (LQTS), mutation sites are reported to correlate with clinical phenotypes in Caucasians, but the relationship in Asian patients remains unknown. The present study was designed to determine whether the location of *KCNH2* mutations would influence the arrhythmic risk in LQT2 patients.

Methods and Results In 118 genetically-confirmed LQT2 patients (69 families, 62 *KCNH2* mutations), the ECG parameters, Schwartz scores, and the incidence of cardiac events, defined as syncope, aborted cardiac arrest, and sudden cardiac death, were evaluated. To examine the effect of mutation sites, the participants were divided accordingly: pore (n=56) and non-pore (n=62) groups. The corrected QT_{end} interval was significantly greater in the pore than in the non-pore group (QTc; 522±63 ms vs 490±49 ms, p=0.002). In this study, the clinical course of each of the probands did not differ according to the mutation sites, whereas non-probands carrying the pore site mutation experienced their first cardiac events at significantly younger age than those with the non-pore site mutation (log-rank, p=0.0005).

Conclusions In a Japanese LQT2 cohort, family members with the pore site mutation were at higher arrhythmic risk than those with the non-pore site mutation. (*Circ J* 2008; **72**: 694–699)

Key Words: Arrhythmia; Long-QT syndrome; QTc interval; Risk factors; Torsade de pointes

The long QT syndrome (LQTS) is an inherited arrhythmogenic disease of the structurally normal heart that may cause sudden death. LQTS is characterized by an abnormality in myocardial repolarization that leads to prolongation of the QT interval, morphological changes in T waves and torsades-de-pointes (Tdp) type of ventricular tachycardia on surface ECGs[1,2] To date, 8 distinct genes responsible for LQTS have been identified, including those of Andersen (LQT7) and Timothy (LQT8) syndromes: on chromosome 11q15.5 (*KCNQ1*; LQT1), 7q35–36 (*KCNH2*; LQT2), 3p21 (*SCN5A*; LQT3), 4q25–27 (*ANKB*; LQT4), 21q22 (*KCNE1*; LQT5), 21q22 (*KCNE2*; LQT6), 17q23 (*KCNJ2*; LQT7) and 12p13.3 (*CACN1C*; LQT8).[3–10]

Moss et al[11] extensively examined the relationships between the site of mutation and clinical phenotype in approximately 44 different LQT2-related *KCNH2* mutations. They reported that subjects with causative mutations in the pore region (n=38, amino acid residues 550 through 650) had more severe clinical manifestations and experienced a higher frequency (74% vs 35%; p<0.001) of arrhythmia-related cardiac events occurring at younger age than did subjects with non-pore mutations (n=166).

In LQT1, based on the United States portion of the International LQTS Registry (n=425), the Netherlands' LQTS Registry (n=93), and the Japanese LQTS Registry (n=82), 600 patients with *KCNQ1* mutations were classified into 2 groups of patients with transmembrane and C-terminus mutations and their clinical phenotypes were examined.[12] That study found that patients with transmembrane mutations were at increasing risk for cardiac events (hazard ratio, 2.06; p<0.001). Shimizu et al also studied the mutation site-dependent differences in 95 LQT1 patients from a multicenter Japanese population and also found that patients with transmembrane mutations were at higher risk of cardiac events and had longer QTc and $T_{peak\text{-}end}$ intervals.[13]

In Japanese LQT2 patients, mutation site dependency is unclear, although this has been reported in Caucasian patients. Therefore, in the present study we aimed to compare the genotype and phenotype relationship, according to the classification adopted by Moss et al,[11] in 118 Japanese LQT2 patients who were genetically identified in the 3 genetic centers in Japan and had no other mutations in LQTS-related genes (except LQT4 and 8).

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Table 1 *KCNH2* **Mutations by Location, Amino-Acid Coding, Type of Mutation, and Reported Functional Effects**

	No. of families	No. of subjects	Position	Exon	Type of mutation	Functional effect in expression studies
Pore regions						
A561T	1	1	S5	7	Missense	Trafficking defect (22)
A561V	1	1	S5	7	Missense	Dominant negative (23)
W563C*	1	1	Pore	7	Missense	
W563G*	1	2	Pore	7	Missense	
C566F*	1	1	Pore	7	Missense	
G572S	2	4	Pore	7	Missense	
M574V*	1	3	Pore	7	Missense	
R582L	1	2	Pore	7	Missense	
R582C	1	1	Pore	7	Missense	
G584C*	1	2	Pore	7	Missense	
G590V*	1	3	Pore	7	Missense	
I593V*	1	1	Pore	7	missense	
K595N*	1	2	Pore	7	Missense	
K595E*	1	1	Pore	7	Missense	
G601S	2	5	Pore	7	Missense	Trafficking defect (22, 24)
G604S	2	2	Pore	7	Missense	
S606P*	1	1	Pore	7	Missense	
T613M	2	3	Pore	7	Missense	Dominant negative (25)
A614V	4	6	Pore	7	Missense	Dominant negative (26)
T623I	1	1	Pore	7	Missense	Trafficking defect (22)
G628S	1	2	Pore	7	Missense	Trafficking defect (22)
N629K	1	1	Pore	7	Missense	Dominant negative (27)
N633S	1	1	Pore	7	Missense	
K638del	1	1	S6	7	Deletion	
F640del*	1	1	S6	7	Deletion	
S641F	1	3	S6	7	Missense	
V644F	1	4	S6	7	Missense	
Subtotal	34	56				
Non-pore regions						
N-terminal regions						
V41A*	1	1	N-term	2	Missense	
Y43D*	1	3	N-term	2	Missense	
E50fs+10X*	1	1	N-term	2	Deletion/frameshift	
G53S*	1	1	N-term	2	Missense	
82-84insIAQ	1	1	N-term	2	Insertion	
F106L*	1	1	N-term	3	Missense	
D111V*	1	1	N-term	3	Missense	
V115M*	1	1	N-term	3	Missense	
P151fs+179X	1	1	N-term	3	Insertion/frameshift	
G187-A190del*	1	3	N-term	4	Deletion	
R312-S318del*	1	2	N-term	5	Deletion	
S320L	1	1	N-term	5	Missense	
P334L	1	1	N-term	5	Missense	
K364fs+3X*	1	3	N-term	5	Insertion/deletion/frameshift	
K386fs+3X*	1	4	N-term	5	Insertion/frameshift	
Transmembrane domains other than pore regions						
Q301X	1	2	S1	6	Nonsense	
F471fs+50X*	1	1	S1-S2	6	Deletion/frameshift	
I489F*	1	1	S1-S2	6	Missense	
A490T	1	1	S1-S2	6	Missense	Current density↓ (28)
H492Y*	1	2	S1-S2	6	Missense	
W497X*	1	3	S3	6	Nonsense	
D501N	1	1	S3	6	Missense	
R534C	1	2	S4	7	Missense	Trafficking defect (22)
C-terminal region						
Q738X*	1	2	C-term	9	Nonsense	
G745-G749del, FinsfS+56X*	1	1	C-term	9	Insertion/deletion/frameshift	
R752W	1	2	C-term	9	Missense	Trafficking defect (22)
S818L	1	1	C-term	10	Missense	Reduced I_{Kr} current (29)
P846T*	1	1	C-term	10	Missense	
W853fs+14X*	1	2	C-term	10	Deletion/frameshift	
R863X	1	2	C-term	10	Nonsense	
L911fs+6X*	1	3	C-term	12	Deletion/frameshift	
R912fs+63X*	1	2	C-term	12	Insertion/frameshift	
S1029fs+23X*	1	3	C-term	13	Deletion/frameshift	
P1034fs+23X*	1	3	C-term	13	Insertion/frameshift	
A1144T*	1	2	C-term	15	Missense	
Subtotal	35	62				

*Novel mutation.
del, deletion; ins, insertion; fs, first amino acid affected by a frameshift (number after fs is number of amino acids before termination); term, terminus.

Table 2 Clinical Characteristics of Pore and Non-Pore Mutations

	Pore (n=56)	Non-pore (n=62)	p value
Demographics			
Female gender (%)	33 (59%)	42 (68%)	0.344
Proband (%)	33 (59%)	34 (55%)	0.712
Age (years) at baseline ECG (range)	31±18 (7–74)	31±16 (2–71)	0.920
Age (years) at first event (range)	16±10 (5–48)	20±13 (5–71)	0.203
Diagnosis			
Schwartz score	5.3±1.6	4.5±1.8	0.017
Schwartz score ≥4 (%)	47 (84%)	41 (66%)	0.034
ECG measurements			
Heart rate (beats/min)	65±13	64±15	0.537
RR (ms)	953±188	975±186	0.510
QT_{end} (ms)	505±79	482±69	0.089
QT_{peak} (ms)	377±67	382±65	0.650
$T_{peak-end}$ (ms)	129±55	99±41	0.001
Corrected QT_{end} (ms)	522±63	490±49	0.002
Corrected QT_{peak} (ms)	389±62	388±47	0.927
Corrected $T_{peak-end}$ (ms)	134±52	101±42	<0.001
Torsade de pointes (%)	17 (30%)	18 (29%)	1.000
T-wave alternans (%)	7 (13%)	4 (7%)	0.346
Notched T wave (%)	43 (77%)	32 (52%)	0.007
Cardiac events			
All cardiac events (%)	38 (68%)	32 (52%)	0.092
Syncope (%)	36 (64%)	32 (52%)	0.194
Aborted cardiac arrest/SCD (%)	6 (11%)	2 (3%)	0.145
Therapy			
β-blocker therapy	26 (53%)	21 (36%)	0.117
Pacemaker (%)	1 (2%)	0	1.000
Sympathectomy (%)	0	0	1.000
Defibrillator (%)	1(2%)	2 (3%)	1.000

Data are mean value ± SD or number (%) of subjects.
ECG, electrocardiography; SCD, sudden cardiac death.

Methods

Study Population

The study population consisted of 118 patients from 69 unrelated Japanese LQT2 families enrolled from 3 institutes in Japan: National Cardiovascular Center, Kyoto University Graduate School of Medicine and Shiga University of Medical Science. The *KCNH2* mutations were confirmed in all patients by using standard genetic tests.[14–17] Screening for mutations in *KCNQ1*, *SCN5A*, *KCNE1*, *KCNE2*, and *KCNJ2* was also conducted, and patients with compound mutations of *KCNH2* and/or additional mutations in these LQTS-related genes were excluded from the analysis. Symptomatic patients were defined as *KCNH2* mutation carriers who experienced at least 1 episode of syncope (ie, complete loss of consciousness, or cardiac arrest requiring cardiac resuscitation), while asymptomatic patients were those without these events. Follow-up was censored at age 50 years to avoid the influence of coronary artery disease on cardiac events.

Genetic Analysis and Characterization

Genomic DNA was isolated from venous blood by use of the QIAamp DNA blood midikit (Qiagen, Hilden, Germany). The protocol for genetic analysis was approved by the institutional ethics committee and was performed under its guidelines. Established primer settings were used to amplify the entire coding regions of the known LQTS genes from genomic DNA.[14–17] Denaturing high-performance liquid chromatography (DHPLC) was used for screening. For aberrant conformers, direct sequencing techniques were performed as described elsewhere.[13] PCR products were denatured at 95°C for 5 min then analyzed by DHPLC. PCR fragments presenting abnormal signals in the DHPLC analysis were subsequently sequenced by the dideoxynucleotide chain termination method with fluorescent dideoxynucleotides in an ABI 3130 genetic analyzer (PE Applied Biosystems).

The pore region of the *KCNH2* channel was defined as the area extending from S5 to the mid-portion of S6 involving amino acid residues 550 through 650, according to a previous report.[11] The non-pore region included the N-terminus region, transmembrane domains other than the pore region and the C-terminus region.

Clinical Characterization

Routine demographic data and basal 12-lead ECGs were obtained for all subjects at the time of enrollment in each institute and there was at least yearly follow-up contact. All ECGs were taken before or without β-blocker medication. The ECG parameters measured from the basal recordings were the RR, QT_{end}, QT_{peak} and $T_{peak-end}$ (QT_{end}–QT_{peak}) intervals. The latter is thought to reflect the transmural dispersion of ventricular repolarization (TDR).[18–20] The rate-dependent QT intervals were corrected for heart rate by Bazett's method.[21] The QT_{peak} was defined as the time interval between QRS onset and the peak of the positive T wave or the nadir of the negative T wave. $T_{peak-end}$ was then obtained by calculating QT_{end} minus QT_{peak}.

These parameters were measured manually in lead V_5 averaged from 2 or 3 consecutive beats. Bifid T waves other than U waves were included in the QT measurements. If ECG recordings were obtained during a cardiac event, the patients were requested to undergo the examination again after improving. Measurements were performed by 3 investigators who were completely unaware of the patient's clinical and genetic status. There were no significant differences in the measured data between the investigators, and the

mean values were used for analysis. LQTS-related cardiac events were defined as syncope, aborted cardiac arrest, or unexpected sudden death.

Statistical Analyses

All data are expressed as the mean value±SD. The Student's t-test was used to compare continuous data between mutations located in the pore region and those in the non-pore region. Differences in frequencies were analyzed by the chi-square test. Time to the first cardiac event (syncope, cardiac arrest, or sudden cardiac death) before initiation of β-blocker therapy and before age 50 years was determined by Kaplan-Meier cumulative estimates. Two-sided probability values <0.05 were considered statistically significant. Statistical calculations were performed with SPSS software (version 11.01J, Chicago, IL, USA).

Results

Genetic Characteristics

Table 1 lists the *KCNH2* mutations we identified, classified by location, number of patients with these causative mutations, coding effects (missense, insertion, deletion and frameshift) and functional outcomes. We identified 62 different *KCNH2* mutations among the 69 LQTS families: 42 missense, 16 deletion/insertion, 11 frameshift and 4 nonsense mutations. There were 27 (44%) mutations causing amino acid changes in the pore region and 35 (56%) mutations within the non-pore regions (15 in the N-terminus, 8 in the non-pore transmembrane, and 12 in the C-terminus). In the pore mutations there were 25 (93%) missense mutations and the remaining 2 were protein deletions (K638del and F640del).

In contrast, the non-pore mutations included more significantly complex mutations such as deletion, insertion, frameshift or nonsense mutations that resulted in truncation of channel proteins (15/35, 43%). Thirty-five mutations (56%, 11 in the pore region and 24 in the non-pore regions) were novel and indicated by asterisk in Table 1. Functional effects by cellular electrophysiologic tests have been reported in only 12 of the 62 mutations (19%);[22–29] however, all those previous reports indicated that the *KCNH2* mutations had loss-of-function effects and made the I_{Kr} current reduce or disappear. Four pore mutations had dominant-negative effects, 4 pore mutations and 2 non-pore mutations had trafficking defects, and 2 non-pore mutations reduced the I_{Kr} current.

Clinical Characteristics

Table 2 is a comparison of the clinical characteristics of the 56 patients with pore mutations and the 62 patients with non-pore mutations. There were no significant differences between the 2 groups regarding gender, the percentage of probands and the age at baseline ECG recording. Diagnostic LQTS scores of Schwartz et al[30] were noticeably greater in the pore group. RR and QT_{peak} intervals were comparable; however, corrected QT_{end} and $T_{peak-end}$ intervals were much longer in the pore than in the non-pore group. Although the incidence of TdP and T-wave alternans did not differ between groups, notched T waves were more frequently seen in the pore group (p=0.007 vs non-pore group). The incidence of cardiac events and the introduction of β-blocker therapy were not statistically different between the 2 groups.

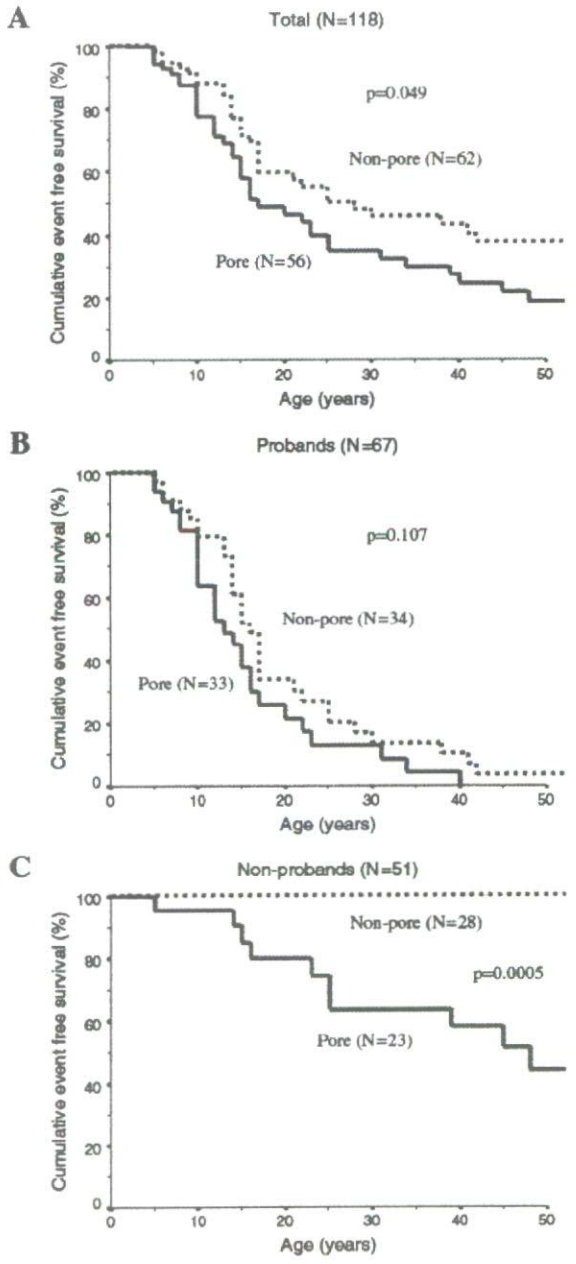

Fig 1. (A) Kaplan-Meier cumulative cardiac event-free survival curves from birth through to age 50 years for the total of 118 patients with *KCNH2* mutations located in the pore (n=56, smooth line) and non-pore (n=62, dotted line) regions. The pore group patients experienced their first cardiac event at a younger age than the non-pore group (log-rank, p=0.049). The difference was caused mainly by the high first-event rate in non-probands. Kaplan-Meier cumulative cardiac event-free survival curves for 67 probands (B) and 51 non-probands (C) with pore mutations (smooth line) and non-pore mutations (dotted line).

Clinical Course by Mutation Location

Fig 1A shows the Kaplan-Meier cumulative cardiac event-free survival curves from birth through to age 50 years for 118 patients (pore group, n=56; non-pore group, n=62). The pore-group patients experienced their first cardiac event at a younger age than the non-pore group (log-rank, p=

Table 3 Clinical Characteristics of Pore and Non-Pore Mutations in Non-Probands

	Pore (n=23)	Non-pore (n=28)	p value
Demographics			
Female gender (%)	14 (61%)	19 (68%)	0.769
Age (years) at baseline ECG (range)	42±20 (9–74)	33±20 (2–71)	0.124
Diagnosis			
Schwartz score	4.7±1.5	3.5±1.7	0.008
Schwartz score ≥4 (%)	18 (78%)	12 (43%)	0.021
ECG measurements			
Heart rate (beats/min)	65±15	70±17	0.251
RR (ms)	959±179	894±179	0.201
QT_{end} (ms)	480±51	441±54	0.0011
QT_{peak} (ms)	352±47	352±53	0.974
$T_{peak-end}$ (ms)	128±46	89±30	0.001
Corrected QT_{end} (ms)	494±45	470±40	0.044
Corrected QT_{peak} (ms)	364±49	374±40	0.423
Corrected $T_{peak-end}$ (ms)	131±43	96±32	0.002
Torsade de pointes (%)	1 (4%)	0	0.451
T-wave alternans (%)	0	0	–
Notched T wave (%)	17 (74%)	14 (50%)	0.095
Cardiac events			
All cardiac events (%)	11 (48%)	0	<0.001
Syncope (%)	10 (43%)	0	<0.001
Aborted cardiac arrest/SCD (%)	1 (4%)	0	0.451
Therapy			
β-blocker therapy	6 (26%)	0	0.006
Pacemaker (%)	0	0	–
Sympathectomy (%)	0	0	–
Defibrillator (%)	0	0	–

Data are mean value ± SD or number (%) of subjects.
Abbreviations see in Table 2.

0.049). We examined the clinical course of the 67 probands and 51 non-probands separately (Figs 1B,C). The clinical courses of the probands were not significantly different according to mutation site (Fig 1B), whereas in the non-pore group 28 non-probands remained asymptomatic and more than half had suffered from cardiac events by the age of 50 (Fig 1C). Therefore, the difference stemmed from markedly distinct prognoses among the non-probands.

Table 3 summarizes the clinical characteristics of the 51 non-probands. The absolute and corrected QT_{end} and $T_{peak-end}$ intervals were all significantly greater in the pore than in the non-pore group. In the non-probands, the incidence of all cardiac events, syncope, and β-blocker therapy were significantly greater in the pore group than in the non-pore group.

Discussion

This study demonstrates that the clinical features of 118 Japanese LQT2 patients who had 62 different *KCNH2* mutations correlated with the mutation sites, but only in non-probands. In probands, there was no significant relationship between mutation site and prognosis. Moss et al[11] reported approximately 179 LQT2 patients based on 44 different *KCNH2* mutations and those patients with pore mutations had significantly ($p<0.0001$) higher frequency of LQTS-related cardiac events and longer QTc intervals than those with non-pore mutations. In contrast to their results, in the present study the mutation-dependent difference in prognosis was relatively small, though significant ($p=0.049$), when analyzed in the total patient cohort (Fig 1A). Indeed, the beneficial outcome of the non-pore patients stemmed from their family members (Fig 1C), and probands showed virtually similar prognosis to that of pore mutation carriers. Although Moss et al did not report separate sub-analysis of probands and family members, the percentage of family members in their non-pore group was significantly larger than that of the pore group (84% vs 57%, $p<0.001$). The very good prognosis of the non-pore mutation group in their study may have reflected that large number of family members.

The character of the mutation per se may be important as another reason for the variance between these 2 studies, as both had a similar number of LQT2 patients. Compared with the study by Moss et al,[11] the type of mutation in the present study was quite different: in our non-pore group, there were significantly more complex mutations, such as nonsense or frameshift, that caused the truncation of channel proteins (15/35, 43%) than in the report of Moss et al (4/30, 13%). For example, nonsense-mediated mRNA decay (NMD) has recently been reported to play an important role in reducing dominant negative suppression effects.[31] Premature termination codon caused by either a deletion or insertion mutation would also cause NMD and thereby attenuate the severity of cardiac phenotypes. This different nature of the mutations may cause the apparently different prognosis of the non-pore mutation groups in each study.

In our pore site mutation group, there were only 2 in-frame deletions, but no frameshift mutations (Table 1). Although it was practically very difficult to conduct every functional assay for each novel *KCNH2* mutation identified here, some cellular electrophysiological effects are available in a small number of *KCNH2* mutations we found (Table 1). Several missense mutations in the pore region (such as A561V and T613M) have been shown to produce dominant negative suppression effects, a greater functional change predisposing to arrhythmic events. In contrast, functional assay of several missense mutations in the non-pore regions has revealed relatively smaller loss-of-function effects (such as with A490T or S818L) Greater functional disruption may also be reflected in the different prognosis

of family members in the pore and non-pore groups (Fig 1).

Previously we reported that LQT1 patients with *KCNQ1* mutations located in the transmembrane regions, including the pore region, are at a higher risk of congenital LQTS-related cardiac events and longer QTc and $T_{peak-end}$ intervals than are patients with C-terminal mutations.[13] In LQT2, we have also demonstrated that $T_{peak-end}$, representing transmural dispersion of ventricular repolarization,[19] is longer in pore patients than in non-pore patients (Table 2), supporting the finding that family members with pore mutations are more likely to suffer from LQTS-related cardiac events than those with a non-pore mutation.

Study Limitations

Cardiac events are not simply linked to the site of mutation in probands; there are other triggering factors such as modifier genes, including single nucleotide polymorphisms,[32] hypokalemia and bradycardia, which play significant roles in aggravating the symptoms of *KCNH2* mutation carriers. The influence of these factors could be interpreted in the similar occurrence of cardiac events in the probands irrespective of mutation site, because the presence of symptoms usually caused the patient to agree to undergo genetic testing.

Regarding each mutation, the number of study patients was relatively small (at most 5), and the location of the mutations was scattered, even in the same pore region. The coding effect was also so various that we had limited ability to show arrhythmic risk according to a specific mutation site. Our cohort contained 35 novel *KCNH2* mutations, and their functional outcomes were not available. Moreover, our study population included only Japanese, so more subjects per mutation and a greater spectrum of *KCNH2* mutations in a worldwide study are needed to evaluate the arrhythmic risks associated with these mutations.

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Age- and Genotype-Specific Triggers for Life-Threatening Arrhythmia in the Genotyped Long QT Syndrome

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Age and Long QT Syndrome. ***Introduction:*** **Patients with long QT syndrome (LQTS) become symptomatic in adolescence, but some become at age of ≥20 years. Since it remains unknown whether clinical features of symptomatic LQTS patients differ depending on the age of onset, we aimed to examine whether triggers for cardiac events are different depending on the age in genotyped and symptomatic LQTS patients.**

Methods and Results: **We identified 145 symptomatic LQTS patients, divided them into three groups according to the age of first onset of symptoms (young <20, intermediate 20–39, and older ≥40 years), and analyzed triggers of cardiac events (ventricular tachycardia, syncope, or cardiac arrest). The triggers were divided into three categories: (1) adrenergically mediated triggers: exercise, emotional stress, loud noise, and arousal; (2) vagally mediated triggers: rest/sleep; and (3) secondary triggers: drugs, hypokalemia, and atrioventricular (AV) block. In the young group, 78% of the cardiac events were initiated by adrenergically mediated triggers and 22% were vagally mediated, but none by secondary triggers. In contrast, the adrenergically mediated triggers were significantly lower in the intermediate group. The percentage of secondary triggers was significantly larger in the older group than in the other two groups (0% in young vs 23% in intermediate vs 72% in older; $P < 0.0001$). Concerning the subdivision of secondary triggers on the basis of genotype, hypokalemia was only observed in LQT1, drugs mainly in LQT2, and AV block only in LQT2.**

Conclusion: **Arrhythmic triggers in LQTS differ depending on the age of the patients, stressing the importance of age-related therapy for genotyped LQTS patients.** *(J Cardiovasc Electrophysiol, Vol. 19, pp. 794-799, August 2008)*

long QT syndrome, genetic test, age, triggers, drugs, hypokalemia, bradycardia

Introduction

The long QT syndrome (LQTS) is a disease entity characterized by an abnormality in the myocardial repolarization that leads to the prolongation of the QT interval, morphological changes in T waves, and torsade de pointes (TdP) type of ventricular tachycardia on surface electrocardiogram (ECG).[1] The prevalence of LQTS is reported as 1 per 5,000 and it induces syncope and sudden cardiac death usually among young people. Up to date, several different genes have been reported to cause the LQTS.[1-3]

Since the first description on two LQTS-related genes (*KCNH2* and *SCN5A*) in 1995,[1] a number of studies have been performed regarding the relationship between genotype and phenotype. In addition to the genetic background predisposing excessive QT prolongation and TdP, many triggers have been known to modify and aggravate the clinical features of LQTS.[4,5] They are, for example, gender (being female), exercise, emotional stress, loud noise, sudden arousal, drugs, hypokalemia, and bradycardia. Some of them are related to the autonomic nervous tone, and it is well known that LQT1 patients are at a higher risk of TdP during exercise and LQT2 patients, in sudden arousal and auditory stimuli.[5-7]

Although many LQTS patients develop symptoms during adolescence, some of them experience the first cardiac event in their adulthood. In order to study the age-related difference in the LQTS phenotype, we aimed to examine whether the above-mentioned triggers for cardiac events are different depending on the age in genotyped and symptomatic LQTS patients.

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Methods

Study Population

The study population consisted of consecutive 145 symptomatic patients (117 probands and 28 family members) of a known genotype (LQT1, LQT2, and LQT3) from 117 unrelated Japanese families out of 343 genotyped patients (185 probands and 158 family members). They were enrolled from three institutes in Japan – Shiga University of Medical Science, National Cardiovascular Center, and Kyoto University Graduate School of Medicine – between 1996 and 2007. Patients with LQT5, LQT6, LQT7 (Andersen-Tawil syndrome), and compound mutations were excluded from the present study. All of the patients experienced cardiac events, and they were associated with, or triggered by, well-defined conditions. LQTS-related cardiac events were defined as syncope (transient and complete loss of consciousness), documented TdP, aborted cardiac arrest, or unexpected sudden cardiac death without a known cause. We excluded the patients who were genotyped but remained asymptomatic. All subjects or their guardians provided informed consent for the genetic and clinical studies according to each institutional review board's guidelines.

The patients were classified into three groups according to the age of first onset of cardiac events: (1) young group (n = 106): patients who experienced their first cardiac event at age of less than 20 years; (2) intermediate group (n = 20): those who experienced their first cardiac event at age of 20-39 years; and (3) older group (n = 19): those who experienced their first cardiac event after age of 40 years.

Clinical Phenotyping

Routine clinical and electrocardiographic (ECG) parameters were acquired at the time of the first examination for the evaluation of LQTS. Measured parameters on the first recorded ECG included QT and R-R interval in milliseconds, with corrected QT interval (QTc) corrected for heart rate (HR) by Bazett's formula.[8] Measurement for ECG parameters was performed manually on lead V5 (if not available on leads II). A cumulative LQTS diagnostic "Schwartz" score (which is derived in part from the QTc, symptoms, and family history) was assigned.[9] In regard to the family history, we defined positive family history as subjects who have relatives with a Schwartz score of ≥4.

Genetic Analysis

Screening for mutations of *KCNQ1*, *KCNH2*, and *SCN5A* was performed using polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP) or denatured high-performance liquid chromatography analyses (dHPLC, WAVE system; Transgenomic Inc., Omaha, NE, USA). For aberrant PCR products, DNA sequencing was conducted with a DNA sequencer (ABI 3130 DNA Sequencer; Perkin Elmer, Foster City, CA, USA).

Genetic mutations of amino acid sequence were characterized by a specific location and coding effect (missense, nonsense, splice site, frameshift, insertion, deletion, and intronic variant). The transmembrane regions of *KCNQ1*, *KCNH2*, and *SCN5A* were defined as six membrane segments (S1 to S6, amino acid residues 112 through 354 for *KCNQ1*, 397 through 666 for *KCNH2*, and 127 through 1771 for *SCN5A*, respectively). They, therefore, included cytoplasmic and extracellular linkers, as well as the pore region. As for LQT1 and LQT2, the pore region was defined as the area extending from S5 to the mid portion of S6 involving amino acid residues 262 through 354 for *KCNQ1* and 550 through 650 for *KCNH2*, respectively.[10-14]

Triggering Factors

We divided the triggers into three categories:(1) adrenergically mediated triggers: exercise, emotional stress, loud noise, and arousal; (2) vagally mediated triggers: rest/sleep; and (3) secondary triggers: drugs, hypokalemia, and AV block. There was a small number of undefined conditions associated with cardiac events, and they were classified as other triggers and excluded for analysis in Figures 1–3.

Statistical Analysis

Data are expressed as the mean value ± standard deviation (SD). The clinical characteristics of the study groups were compared with the chi-square test for categorical variables. For continuous variables, we analyzed the normally distributed data with one-way analysis of variance and non-normally distributed data with Kruskal-Wallis tests. For comparisons between two groups, the Student's *t*-test was used for parametric data and the Mann–Whitney's *U*-test for non-parametric data. Differences were accepted as significant for P value of <0.05.

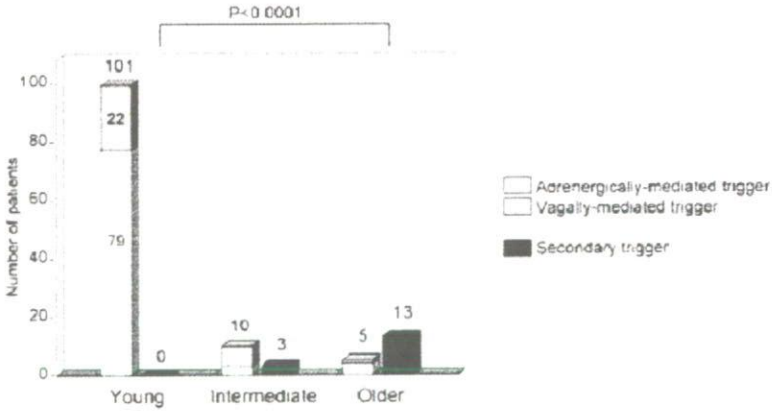

Figure 1. *Triggers for cardiac events in the young, intermediate, and older groups. Incidence of three categorical triggers. Bar graphs show the number of symptomatic patients and their triggers of the first cardiac events: open bars, adrenergically mediated; gray bars, vagally mediated, and black bars, secondary triggers. Other triggers for cardiac events that were undefined were excluded.*