Table 2. Sex-Specific, Age-Adjusted, and Multivariable HRs (95% CLs) for the Incidence of CI and MI According to Dietary Intakes of Soy, Miso Soup, and Beans on the Baseline Questionnaire

	Men				Women			
	Group 1	Group 2	Group 3	*P* for Trend	Group 1	Group 2	Group 3	*P* for Trend
Soy, days per week	0–2	3–4	≥5	...	0–2	3–4	≥5	...
Person-years	63 553	90 453	84 665	...	50 003	94 186	121 378	...
CI								
No. of cases	104	130	153	...	45	65	90	...
Age-adjusted HR	1	0.83 (0.65–1.07)	0.97 (0.76–1.24)	0.853	1	0.77 (0.54–1.12)	0.72 (0.51–1.00)	0.100
Multivariable HR	1	0.85 (0.65–1.11)	0.95 (0.72–1.26)	0.411	1	0.81 (0.55–1.19)	0.64 (0.43–0.95)	0.037
MI								
No. of cases	55	100	87	...	18	26	22	...
Age-adjusted HR	1	1.29 (0.90–1.85)	1.12 (0.77–1.62)	0.663	1	0.61 (0.31–1.18)	0.45 (0.23–0.88)	0.024
Multivariable HR	1	1.26 (0.76–2.07)	1.23 (0.72–2.07)	0.243	1	0.63 (0.31–1.25)	0.55 (0.26–1.09)	0.098
CI and MI combined								
No. of cases	159	230	240	...	63	91	112	...
Age-adjusted HR	1	0.96 (0.78–1.17)	0.96 (0.79–1.17)	0.753	1	0.73 (0.53–1.00)	0.64 (0.47–0.87)	0.008
Multivariable HR	1	1.00 (0.80–1.24)	1.06 (0.84–1.33)	0.570	1	0.81 (0.57–1.16)	0.71 (0.49–1.01)	0.065
Miso soup, times per day	0–1	2	≥3	...	0–1	2	≥3	...
Person-years	83 863	66 778	84 227		105 233	85 645	67 874	
CI								
No. of cases	126	105	152	...	80	55	56	...
Age-adjusted HR	1	0.95 (0.74–1.22)	0.96 (0.76–1.21)	0.848	1	0.74 (0.54–1.02)	0.85 (0.61–1.18)	0.328
Multivariable HR	1	0.83 (0.63–1.11)	0.79 (0.57–1.09)	0.870	1	0.60 (0.40–0.90)	0.58 (0.35–0.96)	0.088
MI								
No. of cases	96	59	82	...	28	22	12	...
Age-adjusted HR	1	0.74 (0.52–1.06)	0.83 (0.60–1.14)	0.248	1	0.97 (0.53–1.78)	0.48 (0.21–1.08)	0.102
Multivariable HR	1	0.99 (0.65–1.51)	1.32 (0.82–2.12)	0.237	1	1.22 (0.55–2.73)	0.44 (0.14–1.32)	0.166
CI and MI combined								
No. of cases	222	164	234	...	108	77	68	...
Age-adjusted HR	1	0.87 (0.71–1.06)	0.90 (0.75–1.08)	0.287	1	0.79 (0.59–1.04)	0.78 (0.58–1.06)	0.108
Multivariable HR	1	0.90 (0.72–1.13)	0.98 (0.78–1.22)	0.889	1	0.74 (0.54–1.03)	0.72 (0.50–1.05)	0.077
Beans, days per week	0	1–2	≥3	...	0	1–2	≥3	...
Person-years	135 265	80 408	22 999	...	137 654	96 610	31 304	...
CI								
No. of cases	226	114	47	...	119	60	21	...
Age-adjusted HR	1	0.84 (0.68–1.05)	1.15 (0.86–1.55)	0.720	1	0.75 (0.56–1.00)	0.62 (0.39–0.97)	0.018
Multivariable HR	1	0.89 (0.71–1.12)	1.25 (0.91–1.71)	0.856	1	0.78 (0.57–1.06)	0.68 (0.42–1.09)	0.055
MI								
No. of cases	146	70	26	...	33	27	6	...
Age-adjusted HR	1	0.84 (0.62–1.14)	0.86 (0.53–1.41)	0.319	1	1.15 (0.66–2.02)	0.72 (0.27–1.87)	0.777
Multivariable HR	1	0.92 (0.67–1.26)	0.98 (0.60–1.62)	0.765	1	1.10 (0.62–1.97)	0.69 (0.26–1.84)	0.676
CI and MI combined								
No. of cases	372	184	73		152	87	27	
Age-adjusted HR	1	0.80 (0.67–0.96)	1.03 (0.81–1.32)	0.363	1	0.83 (0.64–1.06)	0.65 (0.43–0.97)	0.022
Multivariable HR	1	0.86 (0.71–1.04)	1.08 (0.83–1.41)	0.813	1	0.86 (0.65–1.13)	0.71 (0.46–1.09)	0.091

Multivariable HRs were adjusted for age; sex; smoking; alcohol use; body mass index; history of hypertension or diabetes mellitus; medication use for hypercholesterolemia; education level; sports; dietary intake of fruits, vegetables, fish, salt, and energy; menopausal status for women; and PHC.

Table 1 shows CVD risk factors and the intake of selected nutrients and foods, according to the frequency of dietary soy intake. The variables, except for age, were updated according to the 5-year follow-up survey. Compared with persons who ate soy 0 to 2 days per week, on average, those who ate more soy were slightly older and had a lower education level. Those who consumed more soy were also less likely to be current smokers but more likely to be hypertensive. and men

Table 3. Sex-Specific, Age-Adjusted, and Multivariable HRs (95% CLs) for the Incidence of CI and MI According to Dietary Intake of Isoflavones

	Isoflavone Quintiles					
	Q1 (Low)	Q2	Q3	Q4	Q5 (High)	*P* for Trend
Men						
Isoflavone, mean (range), mg/d	10.6 (0–16.4)	18.7 (16.5–21.5)	26.4 (21.6–29.9)	34.7 (30.0–39.5)	45.2 (39.6–87.4)	...
Person-years	47 777	47 781	47 704	47 617	47 737	...
CI						
No. of cases	45	57	70	56	80	...
Age-adjusted HR	1	1.13 (0.81–1.57)	1.14 (0.82–1.59)	0.91 (0.64–1.28)	1.16 (0.84–1.61)	0.046
Multivariable HR	1	1.23 (0.88–1.73)	1.25 (0.89–1.78)	0.95 (0.66–1.37)	1.21 (0.84–1.74)	0.053
MI						
No. of cases	41	49	51	53	48	...
Age-adjusted HR	1	0.72 (0.46–1.13)	1.15 (0.75–1.63)	0.84 (0.56–1.27)	0.74 (0.48–1.14)	0.365
Multivariable HR	1	0.64 (0.41–1.01)	1.02 (0.67–1.56)	0.85 (0.54–1.33)	0.77 (0.47–1.24)	0.614
CI and MI combined						
No. of cases	86	106	121	109	128	...
Age-adjusted HR	1	1.03 (0.80–1.32)	1.08 (0.84–1.37)	0.99 (0.76–1.28)	0.93 (0.70–1.24)	0.639
Multivariable HR	1	1.07 (0.81–1.40)	1.19 (0.90–1.57)	1.10 (0.82–1.48)	1.14 (0.82–1.59)	0.884
Women						
Isoflavone, mean (range), mg/d	11.1 (0–16.2)	18.7 (16.3–23.8)	26.7 (23.9–30.0)	34.3 (30.1–37.7)	41.3 (37.7–73.1)	...
Person-years	52 854	52 871	53 248	53 259	53 233	...
CI						
No. of cases	39	24	31	36	30	...
Age-adjusted HR	1	0.59 (0.37–0.92)	0.61 (0.39–0.95)	0.78 (0.52–1.17)	0.58 (0.37–0.90)	0.315
Multivariable HR	1	0.53 (0.33–0.84)	0.48 (0.30–0.78)	0.55 (0.35–0.87)	0.35 (0.21–0.59)	0.015
MI						
No. of cases	15	22	10	11	8	...
Age-adjusted HR	1	0.82 (0.41–1.64)	0.47 (0.21–1.06)	0.31 (0.12–0.79)	0.36 (0.15–0.86)	0.002
Multivariable HR	1	0.78 (0.38–1.59)	0.46 (0.20–1.07)	0.30 (0.11–0.79)	0.37 (0.14–0.98)	0.006
CI and MI combined						
No. of cases	54	46	41	47	38	...
Age-adjusted HR	1	0.66 (0.47–0.94)	0.55 (0.38–0.79)	0.67 (0.47–0.95)	0.49 (0.34–0.71)	0.001
Multivariable HR	1	0.69 (0.47–1.01)	0.46 (0.30–0.70)	0.60 (0.41–0.89)	0.39 (0.25–0.60)	<0.001

The adjustment variables for HRs were the same as shown in the footnote for Table 2.

in this category were more likely to have diabetes mellitus. The frequency of soy intake was positively associated with total energy intake and with mean daily intake of rice, vegetables, fruits, fish, potassium, calcium, carbohydrate, polyunsaturated fatty acid, saturated fatty acid, fiber, and isoflavones for both sexes. Similar trends were observed according to the frequencies of intake of dietary miso soup and beans.

Table 2 presents hazard ratios (HRs) for the incidence of CI and MI according to the frequencies of dietary intake of soy, miso soup, and beans at the baseline questionnaire. The multivariable HR (95% confidence limits [CLs]) for those who consumed soy foods ≥5 days per week compared with 0 to 2 days per week was 0.64 (0.43 to 0.95, *P* for trend=0.037) for CI, 0.55 (0.26 to 1.09, *P* for trend=0.098) for MI, and 0.71 (0.49 to 1.01, *P* for trend=0.065) for CI and MI combined in women. When we removed potential biological mediators (ie, histories of hypertension and diabetes mellitus and medication use for hypercholesterolemia) from the adjusted variables, we found a weaker but significant inverse association between soy intake and risk of CI and MI combined (Appendix I in the online-only Data Supplement). Similar but weaker inverse associations were observed between intake of miso soup and beans and risk of CI and MI combined. No significant association of dietary intake of soy, miso soup, and beans and isoflavones with CI or MI was present in men.

Table 3 presents HRs for the incidence of CI and MI by quintile of isoflavone intake. An inverse association existed between isoflavone intake and risk of CI and MI in women but not in men. That inverse association became more pronounced after adjustment for confounding variables, in particular, histories of hypertension and diabetes mellitus, and the sex interaction was highly statistically significant ($P<0.001$). The multivariable HRs (95% CLs) for the highest quintile (37.7 to 73.1 mg/d) referenced to the lowest quintile

Table 4. Age-Adjusted Multivariable HRs (95% CLs) of the Incidence of Ischemic CVD According to Dietary Intake of Isoflavones by Menopausal Status in Women

	Isoflavone Quintiles					
	Q1 (Low)	Q2	Q3	Q4	Q5 (High)	*P* for Trend
Isoflavone, mean (range), mg/d	11.1 (0–16.2)	18.7 (16.3–23.8)	26.7 (23.9–30.0)	34.3 (30.1–37.7)	41.3 (37.7–73.1)	...
Premenopausal women						
Isoflavone, mean (range), mg/d	10.8 (0–16.2)	18.6 (16.3–23.8)	26.8 (23.9–30.0)	34.1 (30.1–37.6)	41.1 (37.7–66.6)	...
Person-years	28 021	25 798	24 484	22 606	21 015	...
No. of cases	15	14	14	8	6	...
Age-adjusted HR	1	0.44 (0.20–0.95)	0.52 (0.23–1.19)	0.59 (0.25–1.37)	0.72 (0.32–1.62)	0.463
Multivariable HR	1	0.40 (0.15–1.05)	0.55 (0.22–1.38)	0.60 (0.22–1.38)	0.62 (0.23–1.70)	0.436
Postmenopausal women*						
Isoflavone, mean (range), mg/d	11.3 (0–16.2)	18.8 (16.3–23.8)	26.7 (23.9–30.0)	34.4 (30.1–37.6)	41.3 (37.7–68.0)	...
Person-years	19 957	21 945	23 201	24 876	26 354	...
No. of cases	35	28	24	34	27	...
Age-adjusted HR	1	0.75 (0.47–1.18)	0.58 (0.36–0.92)	0.66 (0.42–1.05)	0.39 (0.23–0.64)	0.002
Multivariable HR	1	0.64 (0.39–1.07)	0.42 (0.24–0.72)	0.50 (0.29–0.85)	0.25 (0.14–0.45)	<0.001

The adjustment variables for HRs were the same as shown in the footnote for Table 2.
*Women with surgical menopause were excluded from the analysis.

(0 to 16.2 mg/d) of isoflavone intake were 0.35 (0.21 to 0.59, *P* for trend=0.015) for CI, 0.37 (0.14 to 0.98, *P* for trend=0.006) for MI, and 0.39 (0.25 to 0.60, *P* for trend <0.001) for CI and MI combined. When we removed the potential biological mediators, we found a weaker but significant inverse association between isoflavone intake and risk of CI and MI combined (Appendix II in the online-only Data Supplement). The inverse association for CI and MI combined was more pronounced in postmenopausal women than in premenopausal women, and the interaction with menopausal status was statistically significant (*P*=0.046; Table 4).

Table 5 presents HRs for ischemic CVD mortality according to the frequencies of dietary intake of soy, miso, and beans reported on the baseline questionnaire. The multivariable HR (95% CL) for those who consumed soy foods ≥5 days per week compared with 0 to 2 days per week was 0.31 (0.13 to 0.74, *P* for trend=0.006) for ischemic CVD mortality in women, but no association was found in men. No significant associations between intake of miso soup and beans and ischemic CVD mortality were present in either men or women.

Table 5. Sex-Specific, Age-Adjusted, Multivariable HRs (95% CLs) of Ischemic CVD Mortality According to Dietary Intake of Soy, Miso Soup, and Beans on the Baseline Questionnaire

	Men				Women			
	Group 1	Group 2	Group 3	*P* for Trend	Group 1	Group 2	Group 3	*P* for Trend
Soy, days per week	0–2	3–4	≥5	...	0–2	3–4	≥5	...
Person-years	62 759	91 002	85 327	...	47 670	92 648	120 147	...
No. of cases	36	70	69	...	14	28	15	...
Age-adjusted HR	1	1.23 (0.82–1.84)	1.21 (0.80–1.82)	0.403	1	1.00 (0.52–1.90)	0.38 (0.18–0.80)	0.004
Multivariable HR	1	1.19 (0.78–1.80)	0.90 (0.56–1.45)	0.202	1	1.15 (0.59–2.24)	0.31 (0.13–0.74)	0.006
Miso soup, times per day	0–1	2	≥3	...	0–1	2	≥3	...
Person-years	85 281	67 946	85 860	...	105 963	86,141	68 361	...
No. of cases	52	42	81	...	25	15	17	...
Age-adjusted HR	1	0.93 (0.62–1.40)	1.33 (0.94–1.89)	0.086	1	0.68 (0.36–1.30)	0.95 (0.51–1.77)	0.791
Multivariable HR	1	0.80 (0.51–1.27)	0.86 (0.53–1.40)	0.598	1	0.89 (0.42–1.88)	0.82 (0.33–2.01)	0.662
Beans, days per week	0	1–2	≥3	...	0	1–2	≥3	...
Person-years	135 174	80 720	23 192	...	134 403	95 216	30 846	...
No. of cases	107	46	22	...	37	11	9	...
Age-adjusted HR	1	0.68 (0.48–0.97)	1.09 (0.69–1.73)	0.464	1	0.40 (0.20–0.79)	0.96 (0.46–1.99)	0.215
Multivariable HR	1	0.75 (0.51–1.10)	1.34 (0.82–2.17)	0.813	1	0.49 (0.24–0.99)	1.22 (0.57–2.61)	0.676

The adjustment variables for HRs were the same as shown in the footnote for Table 2.

Table 6. Sex-Specific, Age-Adjusted, Multivariable HRs (95% CLs) of Ischemic CVD Mortality According to Dietary Intake of Isoflavones

	Isoflavone Quintiles					
	Q1 (Low)	Q2	Q3	Q4	Q5 (High)	*P* for Trend
Men						
Isoflavone, mean (range), mg/d	10.6 (0–16.4)	18.7 (16.5–21.5)	26.4 (21.6–29.9)	34.7 (30.0–39.5)	45.2 (39.6–87.4)	...
Person-years	47 918	47 865	47 790	47 664	47 850	...
No. of cases	25	26	47	35	42	...
Age-adjusted HR	1	0.85 (0.50–1.45)	1.55 (0.98–2.47)	0.97 (0.57–1.66)	1.20 (0.68–2.12)	0.389
Multivariable HR	1	0.86 (0.47–1.56)	1.70 (0.99–2.90)	1.04 (0.57–1.87)	1.27 (0.66–2.43)	0.391
Women						
Isoflavone, mean (range), mg/d	11.1 (0–16.2)	18.7 (16.3–23.8)	26.7 (23.9–30.0)	34.3 (30.1–37.7)	41.3 (37.7–73.1)	...
Person-years	51 874	51 951	52 186	52 285	52 169	...
No. of cases	15	17	9	3	13	...
Age-adjusted HR	1	0.97 (0.49–1.93)	0.71 (0.34–1.48)	0.13 (0.03–0.59)	0.56 (0.21–1.44)	0.010
				0.18 (0.05–0.63)		0.002
Multivariable HR	1	1.26 (0.60–2.65)	1.04 (0.45–2.28)	...*	0.87 (0.29–2.52)	0.103
				0.17 (0.04–0.78)		0.053

The adjustment variables for HRs were the same as shown in the footnote for Table 2.
*Incalculable due to the small number of cases.

An inverse association existed between isoflavone intake and ischemic CVD mortality in women but not in men (Table 6). The multivariable HR (95% CL) of ischemic CVD mortality for the highest to second highest versus the lowest quintiles was 0.17 (0.04 to 0.78, *P* for trend=0.053).

Discussion

In the present study of middle-aged Japanese subjects, we found a significant inverse association between soy and isoflavone intake with the risk of incidence for CI and MI in women but no association in men. The inverse association was primarily observed in postmenopausal women. We also found a significant inverse association of soy intake with the risk of mortality for ischemic CVD in women. To the best of our knowledge, this is the first time such results, for both the incidence of and mortality due to CVD, have been observed in a cohort study.

Soy is the major source of isoflavones in food, with the primary dietary isoflavones being genistein, daidzein, and glycetein.[23] Soy isoflavones can act as antioxidants, reducing the formation of oxidized lipoproteins like LDL.[8] Several articles have shown the reduced oxidative potential of serum in subjects who consume soy protein.[7,8] Isoflavones are structurally similar to estrogens and bind to the estrogen receptor, so it is biologically plausible that they protect against development of atherosclerosis as estrogen agonists.[13,24] We observed a significant risk reduction of CI and MI in the ≥2nd and ≥3rd quintiles of isoflavone intake (≥18 and ≥26 mg/d, respectively) in women. The intake of isoflavones in the 2nd quintile was 20 times greater than the mean levels of isoflavone intake in Westerners.[25] In premenopausal women, estradiol receptors could be occupied with plasma estradiol to a greater extent than in postmenopausal women; therefore, postmenopausal women may benefit more from intake of isoflavones.[13]

Soy contains high polyunsaturated fat (eg, linoleic acid and vitamin E).[2,10] The mechanism by which serum linoleic acid reduces the risk of MI was primarily related to its serum cholesterol–lowering effect.[26] The mechanism for the risk of CI is not clear[27] but could be 1 of the following: dietary intake of linoleic acid may lower blood pressure[28]; it may lower total cholesterol levels[29]; or it may improve glucose tolelance.[30] Soy also contains vitamin E, which may help protect against MI[31] and fatal stroke.[32] Soy also contains n-3 fatty acids, which have been associated with a reduced risk of CVD in several studies.[33,34]

Isoflavones have been the subject of multiple lines of research to identify their impact in terms of their hypocholesterolemic effects,[3,35–38] antioxidant effects,[8] blood pressure–lowering effect,[39] and estrogen-like effects[13,40] on blood vessels. A meta-analysis of 38 controlled clinical studies concluded that daily soy protein consumption resulted in a 9.3% decrease in total serum cholesterol, a 12.9% decrease in LDL cholesterol, and a 10.5% decrease in triglycerides, which are risk factors for coronary heart disease, among hypercholesterolemic subjects but not among subjects with low or normal cholesterol levels.[3] Three recent meta-analyses of the effect of soy protein–containing isoflavones have shown a similar but weak effect. However, the association of soy and isoflavone consumption with a reduced risk of CI may not reflect a direct relationship with the effect of reducing cholesterol levels, because the contribution of hypercholesterolemia to the risk of CI has been weak and inconsistent.[41–44] A study by Ruiz-Larrea et al[45] showed that genistein, the most potent antioxidant of isoflavones in soy protein, enhances resistance of LDL cholesterol to oxidation.

Soy protein with isoflavones might lower blood pressure, unlike casein or milk protein, but only 1 study has shown a significant decrease in blood pressure as a result of consuming soy protein.[39] Most studies indicate that soy and isoflavones do not significantly affect blood pressure.[6]

The present study showed that an increased intake of miso soup by women was associated with a reduced incidence of CI, despite the fact that miso is a major source of salt for most Japanese people. Previous studies have indicated that a higher salt intake increases blood pressure,[46,47] which leads to a higher risk of stroke.[48] Miso soup, however, contains tofu, seaweed, and vegetables, among other ingredients, which may reduce the likelihood of ischemic CVD.[2]

The present study has some methodological strengths compared with previous studies. We evaluated a large prospective cohort enrolled from the Japanese general population. A prospective study has little recall bias, and results from a cohort of general population are more relevant than those of occupational, hospital-based volunteers. We examined the risk of stroke and MI incidence and mortality. Incidence is a more direct measure of stroke and MI risk than death, because the time to CVD death is influenced by treatment. We estimated isoflavone intake using a validated questionnaire. Our sample came from Japanese populations with a large variation in consumption of isoflavones. Associations between exposure and disease can be detected more easily when exposure is variable. Median isoflavone intake among all study participants was 7 times higher than that among Chinese in Singapore[49] and 70 times higher than that among Dutch women.[11] The present study cohort was established in 1990, and thus, we supposed the percentage of isoflavone supplement users to be nearly zero. Finally, we examined menopausal status in the present study, and thus, we could analyze the association of isoflavone intake and incidence of CI and MI stratified by menopausal status.

The present study has several limitations. First, the data for hypertension, diabetes mellitus, and other health conditions were self-reported. These self-reported variables may present a problem with potential misclassification; however, the self-reported data for hypertension and diabetes mellitus may be reasonably accurate, because nationwide annual health screenings have been conducted since 1992 in Japan.[50] Residual and immeasurable confounding is a potential limitation in the present study. No blood pressure measurements or biochemical markers were used in the present study; however, the existence of hypertension and diabetes mellitus was determined from the questionnaires at the baseline survey. Second, measurement errors with nutrient intake are inevitable; however, the reproducibility for soy intake measurements in the baseline and 5-year follow-up questionnaire was good and was compatible with the results reported in the Nurses' Health Study.[33] In the present study, any errors are likely nondifferential and would attenuate associations with soy and isoflavone intake toward a null value. Third, the generalizability to other ethnic groups is uncertain, because few other populations have a large variation of isoflavone intake that allows the examination of an association between isoflavones and disease. Fourth, because we studied dietary intake of isoflavones, the present results are not relevant to the association of isoflavone supplement use with ischemic CVD.

In conclusion, the present community-based prospective study showed that high consumption of isoflavones was associated with a reduced risk of CI and MI among women, particularly postmenopausal women. Our results suggest that the consumption of dietary isoflavones may be beneficial to postmenopausal women for the prevention of ischemic CVD.

Appendix

Study Investigators

The investigators and participating institutions for cohort I of the JPHC Study, a part of the JPHC Study Group (Principal Investigator: S. Tsugane), were as follows: S. Tsugane, T. Hanaoka, M. Inoue, and T. Sobue, Epidemiology and Prevention Division, National Cancer Center, Tokyo; J. Ogata, S. Baba, T. Mannami, Y. Kokubo, and A. Okayama, Division of Preventive Cardiology, National Cardiovascular Center, Osaka; K. Miyakawa, F. Saito, A. Koizumi, Y. Sano, and I. Hashimoto, Iwate Prefectural Ninohe Public Health Center, Ninohe; Y. Miyajima, N. Suzuki, S. Nagasawa, and Y. Furusugi, Akita Prefectural Yokote Public Health Center, Yokote; H. Sanada, Y. Hatayama, F. Kobayashi, H. Uchino, Y. Shirai, T. Kondo, R. Sasaki, and Y. Watanabe, Nagano Prefectural Saku Public Health Center, Saku; Y. Kishimoto, E. Takara, M. Kinjo, T. Fukuyama, and M. Irei, Okinawa Prefectural Ishikawa Public Health Center, Ishikawa; S. Matsushima and S. Natsukawa, Saku General Hospital, Usuda; S. Watanabe and M. Akabane, Tokyo University of Agriculture, Tokyo; M. Konishi and K. Okada, Ehime University, Matsuyama; S. Tominaga, Aichi Cancer Center Research Institute, Nagoya; M. Iida and W. Ajiki, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka; S. Sato, Osaka Medical Center for Health Science and Promotion, Osaka; the late M. Yamaguchi, Y. Matsumura, and S. Sasaki, National Institute of Health and Nutrition, Tokyo; Y. Tsubono, Tohoku University, Sendai; H. Iso and Y. Honda, University of Tsukuba, Tsukuba; H. Sugimura, Hamamatsu University, Hamamatsu; M. Kabuto, National Institute for Environmental Studies, Tsukuba; N. Yasuda, Kochi Medical School; S. Kono, Kyushu University, Fukuoka; K. Suzuki, Research Institute for Brain and Blood Vessels–Akita, Akita; Y. Takashima, Kyorin University, Tokyo; and E. Maruyama, Kobe University, Kobe.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Dietary soy may be beneficial for cardiovascular health because of its high polyunsaturated fat, fiber, vitamin, and mineral content combined with its low saturated fat content; however, few studies have examined the associations of soy and isoflavone intake with risk of cardiovascular disease in terms of both incidence and mortality. Because the Japanese diet is rich in soy, a cohort study of Japanese individuals was warranted. We studied 40 462 Japanese men and women aged 40 to 59 years who were free of cardiovascular disease or cancer at baseline and who answered a food-frequency questionnaire. They were followed up for 12 years to investigate the associations of soy and isoflavone intake with risks of cerebral and myocardial infarction incidence and cardiovascular disease mortality. Soy intake ≥5 times per week versus 0 to 2 times per week was associated with 36% and 69% reduced risks of cerebral infarction incidence and cardiovascular disease mortality in women, respectively. The highest (41 mg/d) versus lowest (11 mg/d) quintile of isoflavone intake was associated with an ≈65% reduced risk of cerebral and myocardial infarction incidence in women. The inverse association of isoflavone intake with risks of cerebral and myocardial infarction incidence was observed primarily among postmenopausal women. No significant association between soy and isoflavone intake and risk of ischemic cardiovascular disease was present in men. Our results suggest that the consumption of dietary isoflavones may be beneficial for postmenopausal women for the prevention of ischemic cardiovascular disease.

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Validation of the Association Between the Gene Encoding Proteasome Subunit α Type 6 and Myocardial Infarction in a Japanese Population

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Background Recently, a large case-control study (2,851 cases and 2,592 controls) reported that a functional single nuclear polymorphism (SNP) in the proteasome subunit α type 6 gene (*PSMA6*) conferred a risk of myocardial infarction (MI) in a Japanese population. The SNP (exon 1, –8C/G) is located in the 5' untranslated region of exon 1, and the risk-conferring allele G appears to enhance the transcription of *PSMA6*, which may exaggerate inflammation through activation of nuclear factor-κ β protein. The frequency of the risk conferring genotype (GG) in cases was reported to be greater than that in controls (12.4% vs 8.9%). The purpose of the present study was to validate this observation in our study population.

Methods and Results Subjects with MI (n=433) were recruited from the outpatient clinic of the National Cardiovascular Center. Control subjects (n=2,186) were recruited from the Suita study. The frequencies of the GG genotype did not significantly differ between the control (9.8%) and MI groups (10.6%). Moreover, this genotype was not associated with C reactive protein levels in the Suita study. However, the GG genotype was significantly associated with greater intima-media thickness (n=2,051, p=0.015) after adjusting for blood pressure, sex, body mass index and age in the Suita study.

Conclusion The reported genotype in *PSMA6* appears not to contribute appreciably to MI, but may contribute slightly to atherosclerosis in the present study population. (*Circ J* 2007; **71:** 495–498)

Key Words: Genetic; Inflammation; Myocardial infarction; *PSMA6*

Myocardial infarction (MI) is a multifactorial disease caused by environmental and genetic factors. There are an increasing number of studies that identify genes that contribute to the incidence of MI; it is possible that these genes can be targeted for personalized prevention of MI[1–3] Recently, a large case-control study (2,851 cases and 2,592 controls) showed that a functional single nuclear polymorphism (SNP) in the proteasome subunit α type 6 gene (*PSMA6*) conferred a risk for MI in a Japanese population[4] The SNP (exon 1, –8C/G) is located in the 5' untranslated region of exon 1, and the risk-conferring allele G appears to enhance the transcription of *PSMA6*, which may increase inflammation through activation of nuclear factor-κ β (NF-κB) protein[5,6] However, because the contribution of a common allele to the pathogenesis of MI appears to be small, validation is necessary in other study populations. The purpose of the present study was to validate the findings of Ozaki et al in a Japanese population and to evaluate the importance of *PSMA6* in the pathogenesis of MI.

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Methods

Study Population

The selection criteria and design of the Suita Study have been described previously[7–9] Genotypes were determined in 2,500 subjects recruited from the Suita Study between April 2002 and February 2004. The MI group consisted of

Table 1 Characterization of Study Population

	Suita study	*MI subjects*	*p value*
Number	*2,186*	*433*	
Male (number)	*992 (45.38%)*	*370 (86.0%)*	*<0.0001*
Age (years)	*5.35±10.90*	*65.85±9.46*	*0.38*
BMI	*22.84±3.34*	*23.74±2.97*	*<0.0001*
HT (%)	*36.37*	*52.42*	*<0.0001*
DM (%)	*19.81*	*41.51*	*<0.0001*
HLP (%)	*62.44*	*73.31*	*0.0004*
TG (mg/dl)	*106.34±68.40*	*127.62±77.31*	*<0.0001*
TC (mg/dl)	*208.97±32.84*	*199.05±39.73*	*<0.0001*
HDL-C (mg/dl)	*60.05±15.41*	*43.91±13.09*	*<0.0001*
Smoking (%)	*15.74*	*57.60*	*<0.0001*
MI (number)	*34 (1.6%)*	*433 (100%)*	

Values are mean ± standard deviation (SD).
MI, myocardial infarction; BMI, body mass index; HT, prevalence of hypertension; DM, prevalence of diabetes mellitus; HLP, prevalence of hyperlipidemia; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; Smoking, current smoking.

Table 2 Association Between MI and rs1048990

Genotype	Suita study*				MI				p value**	p value***
	CC	CG	GG	Total	CC	CG	GG	Total		
Number (%)	1,010 (46.93)	931 (43.3)	211 (9.8)	2,152 (100)	195 (44.3)	192 (45.0)	46 (10.6)	433 (100)	0.73	
Male (%)	44.9	45.5	40.8	44.7	84.6	85.4	89.1	85.5	<0.0001	0.45
Smoking (+) (%)	13.9	17.4	18.0	15.8	53.3	60.4	52.17	57.0	<0.0001	0.97
DM (+) (%)	22.0	17.7	17.1	19.7	38.5	43.2	39.1	40.6	<0.0001	0.089

*Subjects without cardiovascular disease.
**p values are for the comparison between the Suita study and MI subjects.
***p value are for the comparison among genotypes.
Abbreviations see in Table 1.

Table 3 Logistic Analysis of MI

MI	Chi-square	p value	Odds ratio	95%CI
Sex (F)	75.15	<0.0001	0.23	0.16–0.32
Age (years)	7.97	0.0048	3.13	1.42–6.94
Smoking (+)	103.2	<0.0001	3.99	3.06–5.21
Diabetes and/or hyperglycemia (+)	42.77	<0.0001	3.18	2.27–4.56
PSMA6 (GG)	0.02	0.88	0.97	0.63–1.44

Diabetes and/or hyperglycemia (+), subjects diagnosed as having diabetes and/or hyperlipidemia.
CI, confidence interval; PSMA6, proteasome subunit α type 6. Other abbreviation see in Table 1.

Table 4 Association Between *PSMA6* Polymorphism and Intima-Media Thickness

	CC	GC	GG	p value*	p value**
Number	938	884	195		
IMT-mean (mm)	0.79±0.14	0.78±0.13	0.81±0.13	0.025	0.024
Residual IMT-mean	−0.007±0.11	0.005±0.12	0.014±0.11	0.015	0.0073
IMT-max (mm)	1.26±0.53	1.30±0.66	1.24±0.48	0.32	0.38
Residual IMT-max	−0.014±0.469	0.026±0.606	−0.052±0.412	0.099	0.28

Values are mean±SD.
IMT, intima-media thickness. Other abbreviation see in Tables 1,3.
Residuals of IMT were calculated by adjusting for age, systolic blood pressure, sex and BMI.
*p values are for the comparison among CC, CG and GG genotypes.
**p value are for the comparison between CC and GC+GG genotypes.

Table 5 Association Between *PSMA6* Polymorphism and hCRP

	CC	GC	GG	p value*	p value**
Number	1,009	931	210		
hCRP (mg/dl)	0.15±0.47	0.15±0.43	0.11±0.18	0.43	0.69
Log transferred hCRP	−2.79±1.15	−2.76±1.15	−2.80±1.02	0.86	0.71

Values are mean±SD.
hCRP, high sensitivity C related peptide. Other abbreviation see in Table 3.
*p values are for the comparison among CC, CG and GG genotypes.
**p value are for the comparison between CC and GC+GG genotypes.

433 randomly selected inpatients and outpatients with documented MI (370 men, 63 women) who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003[10,11] All subjects enrolled in the present study provided written informed consent. The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center.

Subjects with a systolic blood pressure (SBP) ≥140 mmHg, diastolic blood pressure ≥90 mmHg and/or taking antihypertensive medication were categorized as having hypertension[3] Subjects with a fasting blood glucose ≥126 mg/dl, hemoglobin (Hb) A1c ≥6.5% and/or undergoing treatment for diabetes mellitus (DM) were categorized as having DM[3] Subjects with total cholesterol ≥220 mg/dl, triglycerides ≥150 mg/dl and/or taking antihyperlipidemic medication were categorized as having hyperlipidemia[3] The intima-media thickness (IMT), a well-known indicator of coronary atherosclerosis, was measured on a longitudinal scan of the common carotid artery at a point 10 mm proximal from the beginning of the dilation of the bulb[7]

DNA Study

Ozaki et al determined 8 polymorphisms in *PSMA6* genes, and found the most significant association with MI at the polymorphism rs1048990[4] In the present study, we determined the rs1048990 polymorphism using the TaqMan

methods. The following polymerase chain reaction primer and probe set was used: C_11599359_10 (Applied Biosystems, Foster City, USA).

Statistical Analysis

The values are expressed as mean±standard deviation. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc, Cary, NC, USA). Simple correlation analyses and logistic analyses were performed to determine the association between laboratory data and MI cases. Multiple logistic analyses were performed to obtain predictors for MI. Odds ratio and 95% confidence intervals (CI) were also calculated. The continuous phenotypic variables and genotype were compared using one way analysis of variance, adjusting for appropriate confounding factors. Residuals of IMT were calculated by adjusting for age, SBP, sex and body mass index (BMI)[9] C reactive protein (CRP) levels were logarithmically transformed to attain normal distribution.

Results

The characteristics of the present study population are shown in Table 1. In the present study population, the frequencies of the GG genotype in the MI and the control group were 10.6% and 9.8%, respectively (Table 2). No significant difference was observed in the genotype frequency between the 2 groups. The GG genotype was not associated with smoking habits or the prevalence of DM (Table 2). The odds ratio of the GG genotype of *PSMA6* over the CC+CG genotype for MI was 0.97 (95% CI, 0.63–1.44) (Table 3). However, because it was possible that the sample size of the MI group (n=433) was too small to detect the small effects of the risk-conferring alleles, we observed the effects of this genotype on carotid IMT, an excellent non-invasive marker of atherosclerosis. The GG genotype was associated with mean IMT (p=0.025) and greater residuals of mean IMT (p=0.015) after adjusting for age, BMI, sex and SBP (Table 4).

No significant effects from this genotype on CRP levels were observed in the Suita population (Table 5).

Discussion

The purpose of the present study was to validate in our study population the association between *PSMA6* variants and MI that has been reported in a Japanese population. Because the genetic contribution of a single gene to common disease susceptibility appears to be low, as observed in the insertion/deletion polymorphism of the angiotensin converting enzyme gene in cardiovascular disease, validation studies in other study populations are important![12,13]

PSMA6 encodes the proteasome subunit α type 6, a component of the 20S proteasome![14] The 20S proteasome is composed of 7 α and 10 β subunits, and is the core particle for the 26S ubiquitin-proteasome system, which is important in the regulation of the abundance of proteins involved in various cellular functions, including inflammation![15–17] Of note, this system is involved in the degradation of the IκB protein, which inhibits the activation of NF-κB, a central transcriptional factor that regulates the expression of genes related to inflammation[5] Now vascular inflammation is considered a key player for atherogenesis, and CRP levels are a well-known predictor for subsequent MI![18–21]

The reported odds ratio of the GG genotype of *PSMA6* over the CC+CG genotype for MI was just 1.36 (95% CI, 1.12–1.65)[4] Thus, we were unable to detect the association of the GG genotype with MI, probably due to our small sample size. However, we did detect an influence of this genotype on IMT, a well-known index of atherosclerosis of coronary arteries?[22–25] This may indicate that the influence of this gene may be directed to the pathogenesis of atherosclerosis.

The influence of the *PSMA6* genotype on the residuals of IMT-mean was significant but slight (r^2=0.0042, p=0.014). The IMT-maximum values may be considered to be more influenced by local micro environmental factors and may be difficult to predict using classical risk factors. Indeed, the r^2 values for IMT-maximum by confounding factors (age, gender, SBP and HbA1c) was 0.181, which is smaller than the r^2 values for IMT-mean (r^2=0.237) by confounding factors (age, gender, BMI, SBP and HbA1c). Therefore, a slight influence of the *PSMA6* genotype may not be detected in the IMT-maximum.

Ozaki et al reported that the frequencies of the genotype GG in the MI and the control groups were 12.4% and 8.9%, respectively[4] However, in the present study population, the frequencies of the GG genotype in the MI and the control group were 10.6% and 9.7%, respectively, with no significant differences between groups. Ozaki et al speculated that the effects of *PMSA6* might be due to potentiation of inflammation[4] CRP levels are known to be a good indicator of future MI![20] However, in the present study, the GG genotype was not associated with the CRP levels. The precise mechanism of how the GG genotype might accelerate atherosclerosis or infarction awaits further investigation.

In conclusion, the reported genotype in *PSMA6* appears not to contribute appreciably to MI, but may contribute slightly to atherosclerosis in the present study population.

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REGULAR ARTICLE

Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population

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Abstract

Introduction: Thrombomodulin (TM) is an essential cofactor in protein C activation by thrombin. Here, we evaluated the contribution of genetic variations in the TM gene to soluble TM (sTM) level and deep vein thrombosis (DVT) in Japanese.
Patients and methods: We sequenced the TM putative promoter, exon, and 3′-untranslated region in DVT patients ($n=118$). Among 17 genetic variations we identified, two missense mutations (R385K, D468Y) and three common single nucleotide polymorphisms (−202G>A, 2487A>T, 2729A>C) were genotyped in a general population of 2247 subjects (1032 men and 1215 women) whose sTM levels were measured. We then compared the frequency of these mutations in DVT patients

Abbreviations: DVT, deep vein thrombosis; TM, thrombomodulin; PC, protein C; APC, activated protein C; PS, protein S; EGF, epidermal growth factor; SNP, single-nucleotide polymorphism; sTM, soluble TM; 5′-UTR, 5′-untranslated region; 3′-UTR, 3′-untranslated region.

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with that in the age, body mass index-adjusted population-based controls.
Results: We identified one neutral mutation (H381) and three missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$) of TM in the DVT patients. Age-adjusted mean values of sTM were lower in C-allele carriers of 2729A>C than in noncarriers in the Japanese general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$). Additionally, the CC genotype of this mutation was more common in the male DVT patients than in the male individuals of the general population (odds ratio=2.76, 95% confidence interval=1.14–6.67; $p=0.02$). This mutation was in linkage disequilibrium (r-square>0.9) with A455V mutation.
Conclusions: TM mutations, especially those with a haplotype consisting of 2729A>C and A455V missense mutation, affect sTM levels, and may be associated with DVT in Japanese.

Introduction

Family-based studies have established that venous thromboembolism is, at least in part, an inherited disease with estimated heritabilities of approximately 60% [1,2]. The mode of inheritance of venous thromboembolism is probably complex [2]. Moreover, family-based and twin studies have established that over 25 plasma hemostasis-related analytes (traits) both correlate with thrombosis and are heritable [3–5]. In Caucasians, the factor V-Leiden mutation and prothrombin G20210A mutation are widely recognized as genetic risk factors for deep vein thrombosis (DVT) [6]. However these mutations are not present in the Japanese [7,8]. Recently, we and others found that the protein S (PS) K196E mutation, known as the PS Tokushima mutation, is a genetic risk for DVT in the Japanese population, indicating large differences in the genetics of DVT among ethnicities [9,10].

Thrombomodulin (TM) is a transmembrane protein that is constitutively expressed on the luminal surface of vascular endothelial cells [11]. The anticoagulant function of TM is mediated by interaction with thrombin and protein C (PC). Endothelial membrane-bound TM forms a high-affinity complex with thrombin via thrombin exosite 1, and inhibits thrombin interaction with fibrinogen and protease-activated receptor-1. In contrast, the thrombin–TM complex is a potent activator of PC, and TM enhances thrombin-dependent PC activation by more than two orders of magnitude. Due to the abundance of TM in the microvasculature, the vast majority of thrombin generated under ambient conditions is sequestered by TM. Constitutive inhibition of the procoagulant function of thrombin and tonic formation of activated PC (APC) comprise an essential anticoagulant mechanism that prevents the amplification of thrombin generation, via proteolysis of activated coagulation factors Va and VIIIa by APC.

TM encoded by an intron-less gene consists of a large N-terminal extracellular region, a single transmembrane segment, and a short cytoplasmic tail [12]. The extracellular region is comprised of an N-terminal lectin-like domain followed by six tandem repeats of epidermal growth factor (EGF)-like domains, and a glycosylated (chondroitin sulfate) serine/threonine-rich domain. The thrombin-binding region has been localized to the fifth and sixth EGF-like domains, while the fourth EGF-like domain is required for PC binding to the thrombin–TM complex. The serine/threonine-rich spacer region is required for both thrombin binding and TM cofactor activity for membrane-associated TM. The chondroitin sulfate domain may stabilize thrombin binding to TM, possibly by interacting with the thrombin apolar region [13,14].

Animal model data suggest that TM dysfunction or deficiency is associated with a prothrombotic disorder. Knock-in mice with a TM mutant that has a mutation corresponding to human E387P exhibit a prothrombotic disorder [15]. This amino acid change is located between the interdomain loop of the fourth and fifth EGF-like domains and abolishes the ability of soluble TM (sTM) to catalyze in vitro thrombin activation of PC to APC. Mice with TM deficiency limited to the vascular endothelium die shortly after birth as a result of a consumptive coagulopathy that can be prevented by warfarin anticoagulation [16].

Based on the important antithrombotic role of TM, we hypothesized that genetic variations within the TM gene that alter TM expression and/or impair anticoagulant function could predispose to venous thromboembolism. To test this hypothesis, we screened the promoter, exon, and 3′-untranslated regions (3′-UTR) of the TM gene in unrelated patients with idiopathic, objectively confirmed

DVT for genetic variation. By genotyping three polymorphisms (−202G>A, 2487A>T, 2729A>G) and two missense mutations (R385K, D468Y) in a Japanese general population, we assessed the prevalence of these genetic variations. We then evaluated the association of sTM levels with genetic variations. We finally compared the genotype prevalence of these genetic variations in DVT patients with those in population-based controls to test whether these mutations are associated with DVT in the Japanese.

Patients and methods

DVT patients

A total of 118 Japanese DVT patients (59 men and 59 women, mean age: 52.3 ± 16.1 years old) were recruited from Osaka University Hospital from 2000 to 2004 and the National Cardiovascular Center from 2002 to 2004. All patients examined in this study were unselected patients diagnosed with DVT. Clinical diagnosis of DVT was confirmed by imaging analysis including computerized tomography and ultrasonography.

Screening of genetic variations in TM gene

Blood samples were obtained from DVT patients and genomic DNA was isolated from peripheral blood leukocytes [17]. All the putative promoter, exon, and 3′-UTR regions in 118 Japanese DVT patients were directly sequenced with an ABI PRISM3700DNA analyzer (Applied Biosystems, Foster City, CA) using seven sets of primers. Primer sequences are available upon request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [18]. The A of ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1 [19]. The nucleotide sequence (GenBank Accession ID: AF-495471) was used as a reference sequence.

General population (Suita Study)

The sample selection and study design of the Suita Study have been described previously [20–22]. Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups, underwent a routine blood examination that included lipid profiles and glucose levels, and underwent blood pressure measurements. The basic characteristics of the individuals have been reported previously [23,24]. sTM levels of 2247 population-based samples were measured by an enzyme-linked immunosorbent assay (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Genotyping of mutations and single nucleotide polymorphisms (SNPs) in the general population

Two common SNPs with a minor allele frequency of greater than 5% and all of the missense mutations we detected were tried for genotyping by the

Table 1 Clinical profiles of 118 DVT patients

Clinical profiles		Clinical profiles	
Age, years ± S.D.	52.3 ± 16.1	Nephrotic syndrome, *n* (%)	0 (0.0)
Women, *n* (%)	59 (50.0)	Chronic heart failure, *n* (%)	17 (14.4)
BMI, kg/m^2, mean ± S.D.	23.7 ± 3.2	Diabetes Mellitus, *n* (%)	47 (39.8)
DVT family history, *n* (%)	8 (6.8)	Hyperlipidemia, *n* (%)	48 (40.7)
Previous DVT, *n* (%)	12 (10.2)	Autoimmue disease, *n* (%)	11 (9.3)
		Inflammatory bowel disease, *n* (%)	2 (1.7)
Pregnancy, *n* (%)	5 (4.2)	Estrogen use, *n* (%)	3 (2.5)
Stroke, *n* (%)	1 (1.5)	Steroid use, *n* (%)	9 (7.6)
Prolonged immobility, *n* (%)	14 (11.9)	Paralysis, *n* (%)	5 (4.2)
Malignancy, *n* (%)	16 (13.6)	Myeloproliferative disease, *n* (%)	1 (0.8)
Major surgery (abd, hip, leg), *n* (%)	21 (17.8)	Reduced plasminogen activity, *n* (%)	7 (5.9)
Trauma (pelvis, hip, leg), *n* (%)	3 (2.5)	Reduced antithrombin activity, *n* (%)	7 (5.9)
Stasis due to compression, *n* (%)	6 (5.1)	Reduced protein C activity, *n* (%)	8 (6.8)
Central venous catheter, *n* (%)	0 (0.0)	Reduced protein S antigen, *n* (%)	10 (8.5)
		Lupus anticoagulant (cardiolipin,ACLb2), *n* (%)	3 (11.0)

BMI, body mass index; DVT, deep vein thrombosis; Diabetes mellitus indicates fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication; Hypertension, systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Myeloproliferative disease, Plt. >5 × 10^5 and Ht. >55%; Reduced plasminogen activity, plasminogen activity <70%; Reduced antithrombin activity, antithrombin activity <80%; Reduced protein C activity, protein C activity <70%; Reduced protein S antigen, protein S antigen <60%.

TaqMan-PCR method [25]. Among three missense mutations, genotyping for 1418C>T (A455V) was failed. Additionally, another common SNP (2729A>C) which was in linkage disequilibrium (r-square>0.9) with A455V mutation was genotyped instead of A455V mutation. Thus, five genetic variations were successfully genotyped in 2247 subjects (1032 men and 1215 women). The sequences of PCR primers and probes for the TaqMan-PCR method are available upon request. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of Osaka University Hospital and National Cardiovascular Center. Gene analyses were performed after informed consent had been obtained in written.

Statistical analysis

Values are means ± S.E. The distributions of basic characteristics in men and women in the Japanese general population were examined using the Student's t-test or X^2 analysis. The correlations of two missense mutations and three common SNPs with sTM levels were examined by logistic analysis, with adjustment for confounding factors, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios for each mutation are presented both adjusted for age and age-BMI. All analyses were performed using SAS (release 8.2, SAS Institute Inc.). Statistical significance was established at $p<0.05$. Linkage disequilibrium was calculated using SNPAlyze version 4.0 (DYNACOM Co., Ltd., Mobara, Japan).

Results

Characteristics of DVT patients

The clinical profiles of the 118 Japanese DVT patients (59 men, 59 women aged 52.3 ± 16.1) are summarized in Table 1. Eight patients (6.8%) had a DVT family history and 12 patients (10.2%) had previous DVT. Sixteen patients (13.6%) suffered from cancer and 21 (17.8%) had undergone major surgery of the abdomen, hip or leg. Seven patients (5.9%) had reduced plasminogen activity (<70%) and 7 (5.9%) had reduced antithrombin activity (<80%). Eight patients (6.8%) had reduced PC activity (<70%), and 10 patients (8.5%) had reduced PS antigen (<60%). To eliminate effects of warfarin on PS/PC activities, we did not count numbers of patients having reduced PC activity (PC<70%) and PS antigen (PS<60%) when they had taken warfarin.

Screening of TM gene for sequence variation in DVT patients

On sequencing the TM gene in 118 DVT patients, we identified 17 genetic variants (Table 2). Three of 17

Table 2 Genetic variations in TM gene identified in 118 Japanese DVT patients

SNPs	LD	Region	Amino acid substitution	Allele 1 frequency (%)	Allele 2 frequency (%)	Flanking sequence	db SNP ID
*−832C>A		Promoter		99.6	0.4	gggcagagggcg [c/a] tggtgttaggcc	
*−754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggtttcctga	
*−265C>A		Exon(5′ UTR)		99.6	0.4	aatccgagtatg [c/a] ggcatcagccct	
−202G>A	A	Exon(5′ UTR)		89.2	10.8	ggagggagggcc [g/a] ggcacttataaa	
*−58G>C		Exon(5′ UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gcccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgtt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcggcccttg [c/t] ccgccacattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaaggtg [g/t] acggtggcgaca	
1754C>T		Exon(3′ UTR)		98.7	1.3	aggagcctggct [c/t] cgtccaggagcc	rs13306852
2005G>A	A	Exon(3′ UTR)		89.2	10.8	gtcctcactacc [g/a]ggcgcaggaggg	rs3176134
*2230T>C		Exon(3′ UTR)		99.6	0.4	tcttggtgaatt [t/c] ttttttcctagc	
*2487A>T		Exon(3′ UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3′ UTR)		79.8	20.2	gatgtaaaaggt [a/g] ttaaattgatgt	rs1042580
2729A>C	B	Exon(3′ UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521−3522insT		3′ flanking		99.6	0.4	ctcgggttgtgt [−/t] gtctgttcactt	
*3559T>A		3′ flanking		99.6	0.4	gccctcatttta [t/a] gtcattaaatgg	

LD, mutations in linkage disequilibrium (group A; r-square=0.84, group B r-square=0.93); allele 1, major allele; allele 2, minor allele; *, novel mutation; EGF, epidermal growth factor like domain; Ser/Thr-rich, serine/threonine-rich domain; UTR, untranslated region.

Table 3 Basic characteristics of subjects in general population

	Women (n=1215)	Men (n=1032)	p
Age, years ± S.D.	64.6 ± 10.7	67.1 ± 10.9	<0.0001
Systolic blood pressure, mm Hg ± S.D.	123.5 ± 19.8	126.1 ± 17.9	0.0008
Diastolic blood pressure, mm Hg ± S.D.	74.3 ± 10.4	77.2 ± 10.4	<0.0001
Body mass index, kg/m^2 ± S.D.	22.4 ± 3.2	23.4 ± 3.0	<0.0001
Total cholesterol, mg/dl ± S.D.	215.9 ± 31.6	198.7 ± 31.5	<0.0001
HDL-cholesterol, mg/dl ± S.D.	64.4 ± 15.1	55.2 ± 14.0	<0.0001
Current smokers, %	4.4	27.2	<0.0001
Current drinkers, %	26.0	67.0	<0.0001
Present illness, %			
Hypertension	35.3	42.8	0.0003
Hyperlipidemia	55.7	34.3	<0.0001
Diabetes mellitus	6.1	13.2	<0.0001

Hypertension indicates systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication. The distributions of basic characteristics in men and women in general population were analyzed using the Student's t-test or X^2 analysis.

mutations were missense mutations (R385K; n=2, A455V; n=53 heterozygous, n=14 homozygous, D468Y; n=2). Four mutations within the TM promoter region and the 5′-untranslated region (5′-UTR) (−832C>A, −754G>C, −265C>A, −58G>C) were rare. Twenty-five patients were heterozygous carriers for the −202G>A mutation within the promoter region, which was reported as a −33G>A mutation. This mutation has been reported to decrease TM promoter activity in vitro [26]. It was in linkage disequilibrium (r-square>0.8) with 2005G>A in the 3′-UTR. No patients were carriers for previously reported mutations in the lectin-like domain [A25A (847G>C), E61A (954G>C)] [27,28]. One patient was heterozygous for a novel neutral mutation within the fourth EGF-like domain [H381 (1197C>T)]. Two patients were heterozygous carriers for the previously described R385K mutation (1208G>A) in the fourth EGF-like domain [28]. The previously reported A455V mutation (1418C>T) was found within the sixth EGF-like domain (n=53 heterozygous, n=14 homozygous), an important region for thrombin binding and activation of PC [13]. This mutation was in linkage disequilibrium (r-square>0.9) with the 2729A>C mutation within the 3′-UTR. Within the serine/threonine-rich domain,

Table 4 Genotype distribution of two missense mutations and three common single nucleotide polymorphisms (SNPs) of TM gene in DVT patients and in individuals in general population

SNPs (amino acid change)	Genotypes	Individuals in general population			DVT patients		
		Women	Men	Total	Women	Men	Total
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
−202 G>A	GG	1009 (83.1)	855 (82.9)	1864 (83.0)	45 (76.3)	46 (80.7)	91 (78.5)
	GA	192 (15.8)	157 (15.2)	349 (15.5)	14 (23.7)	11 (19.3)	25 (21.6)
	AA	14 (1.2)	19 (1.8)	33 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1031	2246	59	57	116
1208 G>A (R385K)	GG	1207 (99.3)	1023 (99.1)	2230 (99.2)	57 (98.3)	56 (98.3)	113 (98.3)
	GA	8 (0.7)	9 (0.9)	17 (0.8)	1 (1.7)	1 (1.8)	2 (1.7)
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	58	57	115
1456 G>T (D468Y)	GG	1181 (97.3)	1015 (98.5)	2196 (97.7)	57 (96.6)	57 (100.0)	114 (98.3)
	GT	33 (2.7)	16 (1.6)	49 (2.2)	2 (3.4)	0 (0.0)	2 (1.7)
	TT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1214	1031	2245	59	57	116
2487 A>T	AA	1001 (82.4)	873 (84.6)	1874 (83.4)	41 (83.7)	47 (87.0)	94 (86.2)
	AT	206 (17.0)	155 (15.0)	361 (16.1)	8 (16.3)	7 (13.0)	15 (13.8)
	TT	8 (0.7)	4 (0.4)	12 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	49	54	109
2729 A>C	AA	707 (58.2)	570 (55.2)	1277 (56.8)	24 (43.6)	22 (40.0)	46 (41.8)
	AC	419 (34.5)	393 (38.1)	812 (36.1)	26 (47.3)	25 (45.5)	51 (46.4)
	CC	89 (7.3)	69 (6.7)	158 (7.0)	5 (9.1)	8 (14.6)	13 (11.8)
	Total	1215	1032	2247	55	55	110

Table 5 Comparison of sTM levels by genetic variations of TM gene in general population

SNPs (amino acid change)	Genotypes	Women				Men			
		Age-adjusted		Multi-adjusted		Age-adjusted		Multi-adjusted	
		Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p
−202 G>A	GG	16.9 ± 1.6		17.0 ± 1.6		19.2 ± 1.9		19.6 ± 1.9	
	GA+AA	17.4 ± 0.2	0.73	17.4 ± 0.2	0.77	19.9 ± 0.2	0.68	19.9 ± 0.2	0.87
1208 G>A	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(R385K)	GA+AA	16.2 ± 2.4	0.62	16.0 ± 2.3	0.54	20.5 ± 2.2	0.79	20.4 ± 2.2	0.84
1456 G>T	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(D468Y)	GT+TT	18.1 ± 1.0	0.51	18.1 ± 1.0	0.52	22.2 ± 1.7	0.20	22.6 ± 1.7	0.11
2487 A>T	AA	17.6 ± 0.2		17.6 ± 0.2		20.0 ± 0.2		20.0 ± 0.2	
	AT+TT	16.7 ± 0.4	0.04	16.7 ± 0.4	0.04	19.6 ± 0.6	0.54	19.5 ± 0.6	0.40
2729 A>C	AA	17.9 ± 0.2		17.9 ± 0.2		20.4 ± 0.3		20.3 ± 0.3	
	AC+CC	16.7 ± 0.3	<0.01	16.8 ± 0.3	<0.01	19.4 ± 0.3	0.03	19.5 ± 0.3	0.07

The correlations of five genetic variations with sTM level were examined by logistic analysis, adjusting for age and multiple factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking).

two patients were heterozygous carriers for the previously described D468Y mutation (1456G>T) [29].

Characteristics of individuals in the general population

The characteristics of the 2247 subjects of the Japanese general population group (1032 men, 1215 women) are shown in Table 3. Age, systolic blood pressure, diastolic blood pressure, BMI, percentage current smokers, percentage current drinkers, and frequencies of hypertension and diabetes mellitus were significantly higher in men than in women, while total cholesterol, HDL-cholesterol, and percentage of subjects with hyperlipidemia were significantly higher in women than in men.

Genotyping of two missense mutations (R385K, D468Y) and three common SNPs (−202G>A, 2487A>T, 2729A>C) and association of sTM levels with TM genotypes in the general population

In the general population of 2247 subjects, five mutations were successfully genotyped (Table 4). Plasma levels of sTM were measured in all subjects. As shown in Table 5, sTM levels were significantly lower in C-allele carriers of the 2729A>C mutation than in non-carriers in the general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$), when adjusted for age. Additionally, in male patients, the CC genotype group was associated with significantly higher DVT risk than the combined AA/AC genotype after adjustment for age and age-BMI (odds ratio = 2.76, 95% confidence interval = 1.14–6.67; $p=0.02$ and odds ratio = 2.98, 95% confidence interval = 0.21–7.33; $p=0.02$, respectively) (Table 6). This mutation was in linkage disequilibrium (r-square > 0.9) with the A455V mutation (Table 2).

Discussion

Several mutations within the TM gene have been reported in small numbers of patients with DVT [27,30–33]. However, it was reported that polymorphisms within the TM gene were not common risk factors for incidental DVT in a recent Caucasian population-based case-control study [34]. Because the factor V-Leiden mutation is not detected in Japanese DVT patients [7], while PS Tokushima mutation (K196E) is a risk factor for DVT in a

Table 6 Odds ratios and 95% confidence intervals for DVT in relation to 2729A>C in TM gene

Genotypes	Women				Men			
	Age-adjusted		Age, BMI-adjusted		Age-adjusted		Age, BMI-adjusted	
	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
AA+AC	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
CC	0.97 (0.35–2.70)	0.95	0.96 (0.34–2.70)	0.93	2.76 (1.14–6.67)	0.02	2.98 (0.21–7.33)	0.02

CI, confidence interval.

Japanese population [9,10], we suspected that frequencies of the TM mutations in Japanese DVT patients might differ from those in Caucasians. We therefore performed a case-control study to test TM polymorphisms for associations with DVT in Japanese. In this study, we found that sTM levels were lower in those with 2729C and 2729C was more common in DVT patients than in the general population. It is a reasonable assumption that the low sTM levels in plasma reflect the decreased TM expression on endothelial cells. If so, the capacity of the PC anticoagulant system, which is comprised of TM, PC and PS, would be decreased to thrombosis-prone.

We first screened the TM putative promoter, exon, and 3′ -UTR regions for sequence variations in a random sample ($n=118$) of DVT patients, and identified one novel neutral mutation (1197C>T; H381) and three previously described missense mutations (1208G>A; R385K, 1418C>T; A455V, 1456G>T; D468Y) (Table 2). As shown in previous report showing A455V mutation within the sixth EGF-like domain, an important region for thrombin binding and activation of PC, was a common missense mutation [13], the frequency of A455V mutation was also higher than the other mutation found in this study. The 1197C>T (H381, $n=1$) mutation and 1208G>A (R385K, $n=2$) mutation within the fourth EGF-like domain were rare. Although the fourth EGF-like domain serves as the binding site for PC, the functional consequences of the Arg-to-Lys substitution at position 385 are not known. D468Y mutation lies in the serine/threonine-rich domain. An in vitro study showed that this mutation did not cause any abnormality in levels of production or functional activity of TM [31]. In our study, patients carrying this mutation were rare ($n=2$).

We genotyped five genetic variants in the 2247 population-based controls (Table 4). We failed in genotyping for the A455V mutation, so the 2729A>C mutation in linkage disequilibrium with the A455V mutation was genotyped. In the Japanese general population, the frequency of 2729A>C mutation (36.1% heterozygous, 7.0% homozygous) was higher than that of A455V mutation in Caucasians (24.0% heterozygous, 4.3% homozygous) and African-Americans (15.9% heterozygous, 2.2% homozygous) [33]. Since the frequency of A455V mutation in the Chinese population has been reported to be 45% heterozygous and 9% homozygous [35], the frequency of the 2729A>C mutation in our study was similar to the result in the Chinese population. This difference in genotype frequency may be associated with differences in ethnical genetic background.

The extracellular region of endothelial TM is cleaved and the cleaved fragments are called sTM. sTM processes anticoagulant properties, and sTM levels reported to have a statistically significant correlation with sTM cofactor activity in healthy individuals [36,37]. The LITE Study reported that sTM levels tended to exhibit gene dosage effects, with AA-genotype of A455V mutation carriers exhibiting approximately 10% higher sTM levels than VV-genotype of A455V mutation carriers, and values for the AV-genotype carriers were intermediate, with no significant differences among these three groups [33]. In our study, particularly in women, sTM levels in individuals carrying 2729A>C mutation were lower than those in noncarriers (Table 5). Since the 2729A>C mutation and the A455V missense mutation are in linkage disequilibrium, our findings might support those of these previous reports. For the other mutations, there was no significant difference in sTM level among the genotypes. Despite much interest in sTM as a marker of endothelial injury, few studies have investigated the relationship between sTM and DVT. The findings of previous studies are conflicting or difficult to judge, partly because of small sample sizes or cross-sectional design [33,38–40]. However, systemic infusion of recombinant sTM has been shown to have antithrombotic potential and dose-dependent effects in the prevention of venous thrombosis after total hip replacement [41,42]. Moreover, the ARIC Study, performed in the United States, reported that high levels of sTM are associated with a lower risk of incidental coronary heart disease [43].

Finally, we compared the genotype frequencies in the population-based controls with those in the DVT patients. In male DVT patients, the frequency of 2729A>C mutation was higher than in the population-based controls (Table 6). The LITE Study reported no difference in the frequency of A455V mutation between DVT patients and controls among Caucasians and African-Americans [33]. This discrepancy might come from the difference of sample size, ethnical genetic background or study design. Especially, in our study, difference of mean ages between DVT patients (52.3 ± 16.1 years old) and general population (women: 64.6 ± 10.7 years old, men: 67.1 ± 10.9 years old) may affect the results, although all analysis has been done in age-adjusted manner.

Additionally, significant decrease of sTM levels in the C-allele carriers of 2729A>C mutation was found in women, whereas not much in men in our study (Table 5). However, the incidence of DVT was associated with only men, but not women (Table 6). The mechanisms by which 2729A>C mutation might

contribute to DVT in only men are unknown. This inconsistency might be derived from gender differences or a lack of statistical power due to the sample size. Regarding the gender differences, TM proteins are known to be modulated by estrogens [44]. 17β-estradiol is known to reduce the anticoagulant properties of endothelial cells by decreasing thrombomodulin expression. This can well explain the gender difference of sTM levels, where men showed higher sTM levels than women. The anticoagulant activity of TM was destroyed by oxidation caused by chloramine T, H_2O_2, or hypochlorous acid generated from H_2O_2 by myeloperoxidase [45]. Activated neutrophil, the primary in vivo source of biological oxidants, also rapidly inactivate TM. Oxidation of Met388 in the sixth EGF-like domain was critical for inactivation. Men are supposed to have greater oxidative stress than women. If so, men might be exposed more for DVT risk. Thus, we suppose that the cause of gender difference in relationship between TM polymorphism and DVT may be via the influences of hormonal and environmental effects.

We observed that 2729A>C mutation and A455V mutation are in linkage disequilibrium and 2729A>C mutation is associated with sTM levels and DVT. At present, the causative genetic mutations for this association are not known. A455V mutation may directly affect the expression of TM molecule. 2729A>C mutation in the 3′ -UTR may affect the mRNA stability. TM mRNA is known to be unstable [46], and C-allele may create more unstable mRNA. Two polymorphisms may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. Therefore, additional in vitro studies are required for the identification of the functional genetic variation. Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [47], the association of TM polymorphism to sTM levels and DVT must be reexamined in other populations.

In summary, TM mutations, especially those with a haplotype consisting of 2729A>C and A455V, affect sTM levels, and may be associated with DVT in Japanese.

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