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The association between atherosclerosis and plasma homocysteine concentration in the general population residing on remote islands in Japan

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Although hyperhomocysteinemia is an important and independent risk factor for vascular disease, the relationship between plasma homocysteine concentration (Hcy) and subclinical atherosclerosis in the general population remains controversial.

We screened 1,845 participants who resided on Japanese remote islands and in mainland. Hcy and clinical values were measured, and methylenetetrahydrofolate reductase gene C677T polymorphism (*C677T/MTHFR*), which is an important genetic factor for regulating Hcy, was analyzed. Carotid intima-media thickness (CIMT) and the cardio ankle vascular index (CAVI) were measured to clinically evaluate subclinical atherosclerosis. CAVI had statistically significant association with Hcy (regression coefficient 0.3159, p=0.025), but CIMT was not. Hcy had statistically significant association with age, systolic blood pressure, high-density lipoprotein cholesterol, creatinine, *C677T/MTHFR*, smoking status and alcohol intake. Although *C677T/MTHFR* was not different among residing areas, Hcy was significantly higher on small islands than in other areas even after adjustment for confounding factors.

We found the statistically significant association between Hcy and CAVI in the general population residing on Japanese remote islands, and significant differences in Hcy among residing areas, suggesting strong influence by acquired factors as well as genetic factors.

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1. Introduction

An elevated level of plasma homocysteine concentration (Hcy) has been established as an important and independent risk factor for atherosclerosis and vascular disease.[1] In experimental studies, homocysteine causes damage to the vascular endothelium and the acceleration of platelet aggregation,[2] and it has been reported that hyperhomocysteinemia is caused

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by genetic and acquired factors. An important genetic factor, which mainly regulates the plasma homocysteine level, is the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism (*C677T/MTHFR*). The C-to-T substitution at nucleotide 677 converts an alanine to a valine residue at amino acid position 222. The homozygous form of TT is associated with reduced enzyme activity, resulting in an elevation of Hcy.[3,4] The homozygous TT genotype was shown to be associated with neural-tube defects, coronary heart disease, and stroke.[5] The frequencies of the 677T allele in different ethnic groups were reported to be 0.34 (95% confidence interval, 0.29-0.39) in Caucasians, 0.42 (0.34-0.50) in Japanese, and 0.08 (0.06-0.12) in Africans.[6] As acquired factors, it has been reported that sufficient intake of folic acid alone or in combination with vitamin B_6 and B_{12} was effective in lowering Hcy in any kind of *C677T/MTHFR* genotype.[7]

Recent technological advances in medical equipment have enabled the non-invasive assessment of atherosclerosis at an early stage.[8] High-resolution B-mode ultrasonography provided a non-invasive method for quantifying arterial wall thickening, and it has been shown that carotid intima-media thickness (CIMT) is a strong predictor of cardiovascular disease.[9] In addition, brachial-ankle pulse wave velocity (baPWV), which provides an estimation of arterial stiffness, has been measured in clinical setting as a surrogate marker for atherosclerosis. Several studies have demonstrated a significant correlation between baPWV and CIMT, and reported that baPWV has been associated with cardiovascular disease.[10,11] Recently, a novel index, the cardio ankle vascular index (CAVI), was developed from baPWV. CAVI not only reflects the stiffness of the aorta, femoral artery, and tibial artery, but also reflects systemic arterial sclerosis, including coronary atherosclerosis.[12] CAVI is essentially independent of changes in blood pressure during examination, and was shown to be an appropriate screening tool for atherosclerosis in the general population.[13]

Although numerous studies have suggested that homocysteine might be a modifiable risk factor for cardiovascular disease, the Heart Outcomes Prevention Evaluation Investigators reported recently that supplements combining folic acid, vitamins B_6 and B_{12} lowered Hcy significantly but did not reduce the risk of major cardiovascular events in patients with vascular disease.[14]

In this study, to clarify the relationship between Hcy and clinical markers related to atherosclerosis, including CIMT and CAVI, and to evaluated the difference of Hcy by residing areas, we investigated the general population residing on remote islands and in mainland in Japan who had the same ethnic background.

2. Materials and Methods

2.1 Participants

Nagasaki Prefecture (Japan) has 54 populated remote islands with about 150,000 inhabitants. In consideration of the difference in lifestyles, the investigated field was separated into three areas and defined as follows: the small islands (S island, O island, and K island) with a population of less than 1,000, the large island (F island) with a population of 43,331, and the mainland (U City) with a population of 52,220 (Figure 1) .

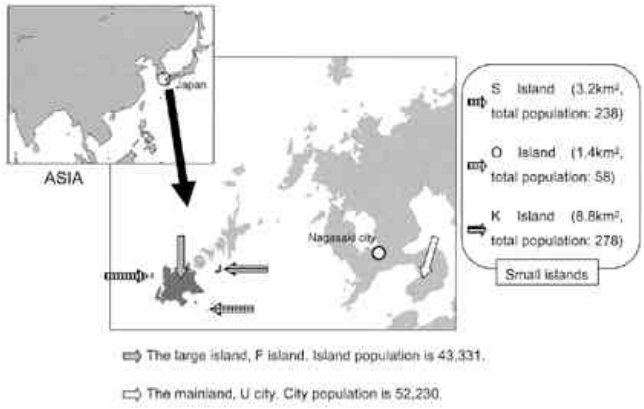

Fig. 1. Map of the research areas. These areas are in Nagasaki prefecture, Japan

Prior to this study, ethics approval was obtained from the special committee of Nagasaki University (project registration number 0501120073). The study subjects were recruited form participants of health examinations in 2005-2006, which were annually conducted by the health center of the local government for residents aged 65 years or over and those aged 40 years or over if covered by the national health insurance. In the Goto city, about 42% of residents had a right to receive the population based survey and 31% of the people were received this survey.

1,716 Japanese adults residing in remote islands participated this study after obtaining informed consent. Among them, 106 participants with an apparent past or present history of cerebral vascular disease, ischemic heart disease, or arteriosclerosis obliterans as atherosclerotic diseases, 46 participants with diabetes mellitus (hemoglobin A_{1C} 6.5% or more), and 4 participants with chronic renal disease (serum creatinine 20 mg/l or more) were excluded from the study. Finally, we enrolled 1,560 participants for further

analysis (1,105 women and 455 men). Of these participants, 134 were residing on the small islands, 1,426 on the large island. Moreover, to compare the difference of Hcy between remote islands and mainland, the 285 (194 women and 91 men) participants residing in the local city in mainland who were recruited from population-based survey were added.

2.2 Data collection and laboratory measurements

The subjects' medical history, alcohol intake (more than once a week vs. none), and smoking status (current smoker vs. non-current smoker) were classified by questionnaire. Height and weight were measured, and body mass index (BMI; kilograms per meter squared) was calculated as an index of obesity. Systolic and diastolic blood pressures (SBP and DBP) were recorded at rest.

Blood samples were collected from each participant after fasting overnight. Serum concentrations of triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), hemoglobin A_{1C} (HbA_{1C}), and creatinine were measured by standard laboratory procedures. Hcy was measured by high-performance liquid chromatography with fluorescence detection.[15]

2.3 Evaluation of atherosclerosis

CIMT of the left and right carotid arteries was measured using LOGIQ Book with a 10-MHz linear array transducer (GE Medical Systems, USA). Subjects were examined in the supine position. On a longitudinal 2-dimensional ultrasonographic image of the carotid artery, the far wall of the carotid artery is displayed as 2 bright white lines separated by a hypoechoic space. The distance from the leading edge of the first bright line (lumen-intima interface) to the second bright line (media-adventitia interface) was identified as the CIMT. Images obtained were analyzed using software Intima Scope® (MEDIA CROSS, Tokyo, Japan). The average of the left and right mean CIMT without part of the plaque was calculated and used for the analysis. To evaluate atherosclerosis precisely, the same technical experts measured CIMT and the same operator analyzed CIMT images using Intima Scope®.

CAVI was recorded using a Vasera VS-1000 vascular screening system (Fukuda Denshi, Tokyo, Japan) with the participant resting in a supine position. The principles underlying CAVI have been described by Yamabe et al.[16] ECG electrodes were placed on both wrists, a microphone for detecting heart sounds was placed on the sternum, and a cuff was wrapped around both the arms and ankles. After automatic measurements, the obtained data were analyzed using VSS-10 software (Fukuda Denshi Co., Tokyo, Japan), and the values of right and left CAVI were calculated. Averages of right and left CAVI were used for the analysis.

2.4 Genotyping of *C677T/MTHFR*

Genomic DNA was extracted automatically from peripheral whole-blood samples using a MagExtractor MFX® (TOYOBO, Osaka, Japan), in accordance with the manufacturer's instructions. To detect C677T transition in the methylenetetrahydrofolate reductase gene, the TaqMan polymerase chain reaction (PCR) method (Applied Biosystems Japan, Tokyo, Japan) was used. In the current analysis, 2 probes were prepared as follows: a C allele-specific probe, 5'-Tet-TCT GCC GcC GAT TTC ATC ATC-Tamra-3', and a T allele-specific probe, 5'-Fam-TCT GCG GGA GtC GAT TTC ATC ATC-Tamra-3'. Primer designs for PCR of the flanking region of *C677T/MTHFR* were as follows: forward, 5'-CTG GGA AGA ACT CAG CGA AC-3'; reverse, 5'-GGA AGG TGC AAG ATC AGA GC-3'. PCR was carried out with a thermal cycler (Bio-Rad Laboratories, Hercules, USA) according to the following conditions: initial denaturation at 95 ℃ for 10 minutes, followed by 35 cycles at 95 ℃ for 15 seconds and 60 ℃ for 60 seconds. The fluorescence level of PCR products was measured using an ABI PRISM7900 Sequence Detector (Applied Biosystems Japan, Tokyo, Japan), resulting in the clear identification of three *C677T/MTHFR* genotypes (CC, CT, and TT).

2.5 Statistical Analysis

Values are expressed as the mean standard deviation or percentage. Differences in each value between female and male were evaluated by Mann-Whitney's U-test or chi-square test. Differences in each value among residents of the small islands, the large island, and the mainland were evaluated by the Kruskal-Wallis test or chi-square test. Multiple regression analysis was performed to clarify the determinants of mean CIMT, CAVI and Hcy, and most appropriate model was selected using stepwise forward procedure. The values are expressed the regression coefficients, the standard errors (SE) and p-values. Hcy in each geographical area and with each *C677T/MTHFR* genotype were compared by analysis of covariance (ANCOVA). Because TG and Hcy levels had skewed distributions, logarithmic transformation was performed. All statistical analyses were performed with SAS software, version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

The characteristics of the 1,560 participants on remote islands are shown in Table 1. All parameters except for SBP and HbA_{1C} were significantly different between women and men. Especially, both CIMT and CAVI were significantly higher in men than in women, and Hcy was also higher in men than in women.

Table 1. Characteristics of the study participants.

	Female (n=1,105)	Male (n=455)	All (n=1,560)
Age (year)*	62.9±10.8	64.2±11.5	63.3±11.0
BMI (kg/m^2)*	22.9±3.3	23.5±3.1	23.1±3.3
SBP (mmHg)	141.2±21.1	142.0±20.4	141.5±20.9
DBP (mmHg) †	83.6±10.9	86.2±11.5	84.4±11.1
TG (g/l) †	1.23±0.72	1.44±0.93	1.29±0.79
HDL-C (g/l) †	0.61±0.15	0.53±0.15	0.58±0.15
LDL-C (g/l) †	1.29±0.33	1.15±0.31	1.25±0.33
HbA_{1C} (%)	5.0±0.4	5.1±0.4	5.1±0.4
Creatinine (mg/l) †	6.6±1.3	8.7±1.9	7.2±1.8
Homocysteine (μmol/l) †	8.9±4.1	10.7±4.6	9.4±4.4
Mean CIMT (mm) †	0.71±0.15	0.74±0.17	0.72±0.16
CAVI †	8.2±1.1	8.5±1.3	8.3±1.2
Current smoker (%) †	4.0	24.0	9.8
Alcohol intake (%) †	12.4	50.1	23.4

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CIMT, carotid intima-media thickness; CAVI, cardio ankle vascular index
*p<0.05, † p<0.001

Hcy had statistically significant association with CIMT (r=0.14, p<0.001) and CAVI (r=0.15, p<0.001) by simple regression analysis (Figure 2). Furthermore, to clarify the

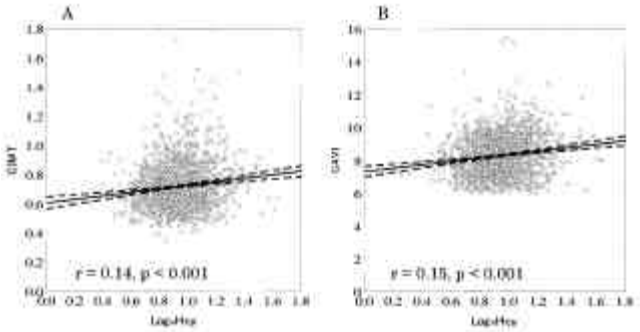

Fig. 2. Relationship between CIMT - homocysteine **(A)**, CAVI - homocysteine **(B)**.

association between Hcy and CIMT or CAVI including confounding factors, we analyzed using multiple regression analysis (Table 2). Mean CIMT had statistically significant association with age (p<0.001), SBP (p<0.001), creatinine (p<0.001), and current smoking (p=0.0278), but not with Hcy. CAVI had statistically significant association with age (p<0.001), sex (p=0.007), BMI (inversely, p<0.001), SBP (p<0.001), TG (p<0.001), HbA1c (p=0.031), Hcy (p=0.025), and alcohol intake (p=0.002). Eventually, Hcy had statistically significant association with CAVI, but not with mean CIMT.

Next, we focused on the differences of Hcy and other parameters by residing areas. We added 285 participants in the local city in mainland and analyzed the difference among the three areas (Table 3). All parameters except for HDL-C, LDL-C, HbA_{1C} and *C677T/NTHFR* genotype were significantly different among the three areas. Especially, although there were no significant differences in the *C677T/NTHFR* genotype, Hcy was significantly higher in participants from the small islands, followed by those from the large island

Table 2. Multiple regression analysis and stepwise procedure for the determination of CIMT and CAVI.

	CIMT (n=1,560)			CAVI (n=1,560)		
	coefficient	S.E.	*p-value*	coefficient	S.E.	*p-value*
Age	0.0061	0.0003	*p<0.001*	0.0593	0.0024	*p<0.001*
Sex	-	-	-	0.1631	0.0600	*p=0.007*
BMI	-	-	-	-0.0355	0.0081	*p<0.001*
SBP	0.0011	0.0002	*p<0.001*	0.0066	0.0013	*p<0.001*
TG	-	-	-	0.4592	0.1130	*p<0.001*
HDL-C	-	-	-	-	-	-
LDL-C	-	-	-	-	-	-
HbA_{1C}	-	-	-	0.1352	0.0628	*p=0.031*
Creatinine	0.0117	0.0019	*p<0.001*	-	-	-
Homocysteine	-	-	-	0.3159	0.1408	*p=0.025*
Current smoker	0.0251	0.0114	*p=0.028*	-	-	-
Alcohol intake	-	-	-	0.1934	0.0631	*p=0.002*

SE: standard error.
Mean IMT: r_2=0.29, CAVI: r_2=0.38

Table 3. Characteristics of the study participants in each residing areas.

	Small islands (n=134)	Large island (n=1,426)	Mainland (n=285)
Age (year) †	67.7±13.7	62.9±10.6	61.0±10.4
Male/female (%)*	40.3/59.7	28.1/71.9	31.9/68.1
BMI (kg/m2)*	23.8±4.0	23.0±3.2	23.3±2.9
SBP (mmHg) †	148.8±20.6	140.8±20.8	128.7±18.42
DBP (mmHg) †	85.9±11.2	84.2±11.1	77.5±10.2
TG (g/l) †	1.27±0.75	1.29±0.80	1.09±0.69
HDL-C (g/l)	0.57±0.16	0.58±0.15	0.58±0.14
LDL-C (g/l)	1.24±0.33	1.25±0.33	1.22±0.27
HbA_{1c} (%)	5.1±0.4	5.1±0.4	5.1±0.4
Creatinine (mg/l)*	6.8±1.9	7.2±1.8	7.0±1.7
Homocysteine (μmol/l) †	11.5±5.1	9.2±4.2	6.5±2.7
C677T/MTHFR(CC/CT/TT,%)	39.5/49.3/11.2	43.8/46.0/10.2	41.7/48.1/10.2
Current smoker (%) †	18.7	9.0	7.7
Alcohol intake (%) †	20.1	23.7	37.9

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *C677T/MTHFR*, methylenetetrahydrofolate reductase gene C677T polymorphism.
*p<0.05, † p<0.001 among three areas

and from the mainland in this order (Figure 3).

In all participants, Hcy with the TT genotype (10.4 ± 5.8 μmol/l, mean ± standard deviation) was significantly higher than with CC (8.6 ± 3.8 μmol/l) or CT genotypes (9.0 ± 4.2 μmol/l) (data not shown). As there was no difference between CC and CT genotypes, we analyzed CC and CT genotypes as one group. To determine the confounding factors of Hcy, we analyzed clinical parameters using multiple regression analysis and stepwise procedure (Table 4). Hcy had statistically significant association with age

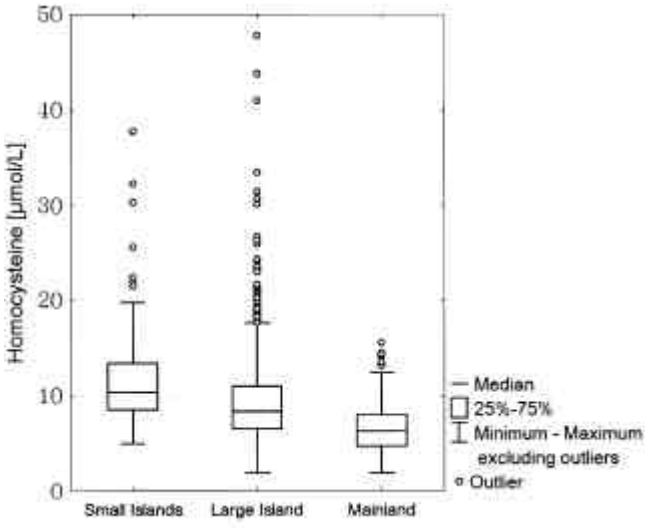

Fig. 3. Plasma total homocysteine among small islands, large island and mainland.

(p<0.001), SBP (p<0.001), HDL-C (inversely, p<0.001), creatinine (p<0.001), *C677T/MTHFR* (p<0.001), current smoking (p<0.001), and alcohol intake (inversely, p=0.019).

According to the ANCOVA after adjustment for age, SBP, HDL-C, creatinine, current smoking, and alcohol intake, adjusted mean of Hcy with all genotypes was significantly different among the three areas. Because there was no difference in the rate of *C677T/MTHFR* genotypes, the *C677T/MTHFR* was excluded from adjustment factors. Hcy on the small islands was the highest, followed by those on the large island and those in the mainland in this order (Table 5). Furthermore, when groups were separated by geographical location and genotype, Hcy on the small islands was the highest among the three areas in both TT and CC&CT genotypes (Table 5). Interestingly, no differences in Hcy were observed between TT and CC&CT genotypes in the mainland, whereas significant differences in Hcy were observed on the remote islands.

Table 4. Multiple regression analysis and stepwise procedure for the determination of plasma homocysteine concentration.

	coefficient	S.E.	*p-value*
Age	0.0015	0.0004	*p<0.001*
Sex	-	-	-
BMI	-	-	-
SBP	0.0010	0.0002	*p<0.001*
TG	-	-	-
HDL-C	-0.1150	0.0277	*p<0.001*
LDL-C	-	-	-
HbA1c	-	-	-
Creatinine	0.0261	0.0021	*p<0.001*
C677T/MTHFR(CC&CT vs. TT)	0.0567	0.0132	*p<0.001*
Current smoker	0.0850	0.0141	*p<0.001*
Alcohol intake	-0.0227	0.096	*p=0.019*

r^2=0.16

Table 5. Plasma homocysteine concentration among residing areas and *C677T/MTHFR* genotypes adjusted for age, SBP, HDL-C, creatinine, current smoker, and alcohol intake.

C677T/MTHFR	Small islands (n=134)	Large island (n=1,426)	Mainland (n=285)
CC&CT (n=1,655)	1.00 (0.02)* †	0.92 (0.01)* †	0.80 (0.01)*
TT (n=190)	1.18 (0.04)* †	0.98 (0.01)* †	0.77 (0.03)*
All genotypes	1.02 (0.01)*	0.93 (0.01)*	0.79 (0.01)*

Vales are adjusted means (standard errors) of logarithmic transformed homocysteine.
*p<0.001 small islands vs. large island, small islands vs. mainland, and large island vs. mainland
† p<0.001 CC&CT vs. TT

4. Discussion

In this study, we screened Hcy and clinical parameters for atherosclerosis in the general population residing on Japanese remote islands and showed that Hcy had statisti cally significant association with CAVI, but not with CIMT. In recent studies, the relationship between Hcy and clinical parameters for atherosclerosis, including CIMT and CAVI, was still unclear. In population-based surveys, Adachi et al. reported that total plasma homocysteine level was significantly related to IMT after adjustment for confounding factors in Japanese general population,[17] and the same association was reported in several countries.[18,19] However, Inamoto et al. reported that total plasma homocysteine level was not associated with either mean or maximum CIMT in similar Japanese general population,[20] and Durga J ea al. concluded in systemic review that the association between Hcy and CIMT in the general population was often weak or absent.[21] Because the measurement method for CIMT was not standardized, various CIMT methodologies were employed for past studies. In addition to the difference of measurement method for CIMT, the heterogeneity in the study population, inclusion criteria, and residing environment might have caused these different results.

Also, the association between Hcy and baPWV is still controversial. Mayer et al reported that baPWV was positively correlated with total plasma homocysteine concentration even after adjustment for conventional cardiovascular risk factors in general population-based study.[22] On the other hand, de Bree conducted that total plasma homocysteine concentration was not associated with baPWV or CIMT in the cross-sectional survey in French population.[23] In our study, CAVI, which was newly developed as an alternative to baPWV, was significantly correlated with Hcy. In experimental studies, it has been shown that homocysteine caused direct injury to vascular endothelial cells and endothelial dysfunction.[24,25] Homocysteine induced oxidative stress in the endothelium and reduced the levels of endothelium-derived vasodilators such as nitric oxide and prostacyclin.[26,27,28] Kobayashi et al reported that baPWV significantly associated with flow-mediated dilation, which is known as a direct marker of nitric oxide bio-availability and as a new evaluation method for vascular endothelial function.[29] Moreover, the homocysteine-lowering treatment improved the endothelial function, which was measured by flow-mediated vasodilation.[30] These results suggest that CAVI might be more sensitive for the influence of homocysteine than CIMT.

We analyzed the confounding factors of Hcy using multiple regression analysis and stepwise procedure, and showed that age, SBP, HDL-C, Creatinine, *C677T/NTHFR* genotype, current smoking, and alcohol intake had statistically significant association with Hcy in our study population. It is well known that the plasma homocysteine level is associated with the major components of cardiovascular risk factors, including aging, HDL-C, and smoking;[31,32] however, the association between Hcy and blood pressure is controversial. In the Framingham study, Jacques et al. reported no association between Hcy and SBP.[21] On the other hand, several recent studies reported that Hcy correlated significantly with SBP in young African Americans and healthy Italian adults.[33,34] Unlike the Framingham study, the present study showed that Hcy had statistically significant association with SBP in Japanese adults. This finding might be caused by differences in ethnic background, the rate of senior participants, and the rate of patients with hypertension.

After adjustment for age, SBP, HDL-C, creatinine, cur rent smoking, and alcohol intake, Hcy was still significantly higher in the order of the small islands, the large island, and the mainland. To the best of our knowledge, this is the first report to show significantly higher Hcy on remote islands compared with the mainland. In the Chinese general adult population, Sakuta et al. reported that Hcy was significantly higher in urban areas than in rural areas in the northern region of China, and the prevalence of hyperhomocysteinemia was higher in urban areas than in rural areas after adjustment for region, age, sex, and season.[35] On the other hand, Hcy was higher in rural Gambia than in the UK;[36] however, these epidemiological reports did not show a difference in the *C677T/MTHFR* genotype. The homozygous form of TT is associated with reduced enzyme activity, resulting in an elevation of Hcy. The present study demonstrated a difference in Hcy among residing areas, although inhabitants in investigated areas were of the same ethnic background and had no significant difference in *C677T/MTHFR*, indicating that Hcy was strongly regulated by acquired factors as well as genetic factors.

In all participants, Hcy with the TT genotype of *C677T/MTHFR* was significantly higher than with CC or CT genotypes in this study. According to meta-analysis regarding homocysteine and stroke, the weighted mean difference in Hcy between the CC and TT genotypes of *C677T/MTHFR* was 1.93 mol/L (95%C.I. 1.38-2.47; $p<0.0001$) without cardiovascular disease.[37] Likewise, the mean difference in Hcy between CC and TT genotypes was 1.83 mol/L (1.16-2.51; $p<0.001$), and there were no significant differences in Hcy between CC and CT genotypes in our study.

Furthermore, the most significant difference in Hcy was found between TT and CC&CT genotype on the small

islands and to a lesser degree on the large island but not in the mainland. Comparing Hcy with the same genotype, there were significant differences among the three areas. These results suggested that acquired factors influenced more strongly than genetic factors. Jacques *et al*. reported that dietary intakes of folic acid, vitamin B_6, and riboflavin were negatively associated with Hcy among non-supplement users, and Hcy was positively associated with alcohol intake, caffeine intake, and antihypertensive medicine use. In addition, several reports showed that physical exercise was associated with lowering Hcy.[31, 38, 39] Because inhabitants on the small islands were surrounded by the sea, and had restricted public services, they might have unique lifestyles different from urban areas, especially in terms of daily movement and dietary habits. Further investigations of lifestyle among the three areas are needed to clarify the association between Hcy and acquired factors.

In conclusion, we demonstrated a statistically significant association between Hcy and CAVI in the general population residing on Japanese remote islands, and significant differences in Hcy among residing areas. To clarify the determinants of Hcy, additional investigations for other factors, such as nutrition and lifestyle, should be carefully considered.

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