Effects of Nitric Oxide Synthase 3 Gene Polymorphisms on Cardiovascular Events in a General Japanese Population
— The Yamagata (Takahata) Study —

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Background: Single nucleotide polymorphisms (SNPs) in nitric oxide synthase 3 (*NOS3*) are associated with cardiovascular risk factors. However, it is not clear whether the *NOS3* SNP is a genetic risk factor for cardiovascular diseases.

Methods and Results: This prospective cohort study included 2,726 subjects aged ≥40 years who participated in a community-based health checkup. We genotyped 639 SNPs, including 2 *NOS3* SNPs (rs1799983 and rs1808593). All subjects were monitored prospectively over a median follow-up period of 16.0 years, with the endpoint being cardiovascular events, including cardiovascular death and/or non-fatal myocardial infarction. Kaplan-Meier analysis demonstrated that both rs1799983 GT/TT and rs1808593 GG carriers had a higher risk of the endpoint than non-carriers. Univariate and multivariate Cox proportional hazard regression analyses revealed that both rs1799983 GT/TT and rs1808593 GG were independently associated with cardiovascular events after adjusting for confounding risk factors. The net reclassification index and integrated discrimination index were significantly improved by the addition of *NOS3* SNPs as cardiovascular risk factors.

Conclusions: *NOS3* gene polymorphisms could be genetic risk factors for cardiovascular events in the general Japanese population, and could be used to facilitate the early identification of individuals at high risk of cardiovascular events.

Key Words: Cardiovascular death; rs1799983; rs1808593; Single nucleotide polymorphism

Cardiovascular diseases remain a major cause of deaths globally.[1,2] Multiple genetic and cardiovascular risk factors affect the development of cardiovascular diseases.[3,4] Single nucleotide polymorphisms (SNPs) are the most frequent genetic variations of DNA.[5] However, it is not clear whether SNPs can be used to screen genes that increase the risk of cardiovascular diseases.

Nitric oxide synthase 3 (NOS3), first identified in endothelial cells, is an enzyme that produces nitric oxide (NO).[6] NO is a small gaseous and lipophilic molecule that plays key roles in the regulation of cardiovascular homeostasis by modulating vascular tonus, inhibiting platelet aggregation, and scavenging superoxide anion.[7,8] Because NOS3 is the most important NO synthase isoform in the vascular endothelium, it plays a critical role in the cardiovascular system. The *NOS3* gene is located in the 7q35–7q36 region of chromosome 7 in humans and is a highly polymorphic gene.[7] Associations between *NOS3* SNPs and cardiovascular risk factors such as hypertension, diabetes, and obesity have been demonstrated.[7] Several cross-sectional studies have examined the association of various *NOS3* SNPs with cardiovascular disease.[9,10] In addition, Severino et al reported that the *NOS3* SNP rs1799983 is associated with ischemic heart disease.[11] However, there has been no prospective cohort study into the association between *NOS3* SNPs and cardiovascular diseases. Thus, the present prospective observational study was in an apparently healthy population. The aim of this study was to examine whether *NOS3* SNPs are associated with future cardiovascular events in the general Japanese population.

Figure 1. Study protocol. NOS3, nitric oxide synthase 3; SNP, single nucleotide polymorphism.

Methods

Ethics Statement

As reported previously,[12] the Yamagata (Takahata) Study was approved by the Ethics Review Committee of Yamagata University Faculty of Medicine (No. 2020-414), and all participants provided written informed consent. All procedures were performed in accordance with the Declaration of Helsinki.

Study Population

This study included 2,946 subjects aged ≥40 years (1,334 men, 1,612 women) who participated in a community-based health checkup program between 2004 and 2005 in Takahata Town. Of the 2,946 subjects, 220 were excluded because of incomplete data: lack of rs1799983 genotype data in 28 subjects, lack of rs1808593 genotype data in 11 subjects, lack of blood pressure data in 4 subjects, and a lack of biochemical markers in 177 subjects. This left 2,726 subjects in the present study.

Follow-up Period and Endpoint

All subjects were prospectively monitored over a median follow-up period of 16.0 years. The endpoint was cardiovascular events, including cardiovascular death and/or non-fatal myocardial infarction. The cause of death was confirmed by death certificates based on the International Classification of Diseases, 10th Revision (ICD-10). Cardiovascular death was defined as death due to diseases of the circulatory system (ICD-10 codes I00–I99), such as coronary artery disease, valvular heart disease, heart failure, arrhythmia, cerebrovascular diseases, or aortic artery disease. Non-fatal myocardial infarction was determined by reviewing the Yamagata Acute Myocardial Infarction Registry data, which covers all acute myocardial infarctions that occur in Yamagata Prefecture.[13] In the present study we used the first event during the follow-up period.
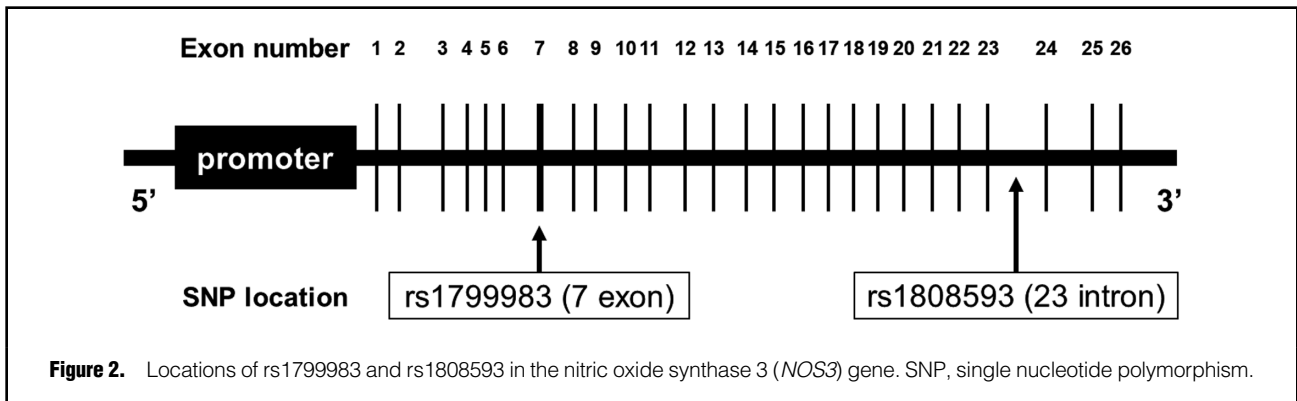
Genotyping

Genotyping was performed using the Invader assay (Third Wave Technologies, Madison, WI, USA) and TaqMan allelic discrimination assay. Reagents were purchased from Applied Biosystems (Foster City, CA, USA). TaqMan probes that can distinguish SNPs after a polymerase chain reaction (PCR) were designed and synthesized by Applied Biosystems. One allelic probe was labeled with the fluorescent FAM dye and the other was labeled with the fluorescent VIC dye.

PCRs were conducted using the TaqMan Universal Master Mix with primers at a concentration of 225 nM and TaqMan MGB probes at a concentration of 50 nM. The reactions were conducted in 382-well plates in a total volume of 3 μL using 3.0 ng genomic DNA. The plates were then placed in a GeneAmp PCR system 9700 (Applied Biosystems) and heated at 95°C for 10 min, followed by 40 cycles at 92°C for 15 s and at 60°C for 1 min, with a final incubation at 25°C. The fluorescent intensities of each well in the plates were read using Prism 7900HT (Applied Biosystems). Fluorescent data files from each plate were analyzed using the SDS 2.0 allele calling software (Applied Biosystems). Several data points were eliminated to preserve the reliability of the assay system (missing data due to poor signal intensity <1.1%).[12,14]

Biochemical Markers

Blood samples were obtained to measure B-type natriuretic peptide (BNP) concentrations. These samples were transferred to chilled tubes containing 4.5 mg EDTA disodium salt and aprotinin (500 U/mL), and centrifuged at 1,000 g for 15 min at 4°C. The supernatant (plasma) was collected and stored frozen at −70°C until analysis. BNP concentrations were measured using a commercially available radioimmunoassay specific for human BNP (Shiono RIA BNP assay kit; Shionogi, Tokyo, Japan).[12,15]

The estimated glomerular filtration rate (eGFR) was

Figure 2. Locations of rs1799983 and rs1808593 in the nitric oxide synthase 3 (*NOS3*) gene. SNP, single nucleotide polymorphism.

Table 1. Baseline Characteristics and Comparisons of Clinical Characteristics Among rs1799983 Genotypes				
Variables	All subjects (n=2,726)	GG carriers (n=2,356)	GT/TT carriers (n=370)	P value
Age (years)	63±10	63±10	63±10	0.708
No. males/females	1,232/1,494	1,067/1,289	165/205	0.803
Previous heart failure	98 (3.6)	82 (3.5)	16 (4.3)	0.418
Family history of CVD	466 (17.1)	406 (17.2)	60 (16.2)	0.629
Atrial fibrillation	37 (1.4)	32 (1.4)	5 (1.4)	0.992
Smoking	880 (32.3)	760 (32.3)	120 (32.4)	0.947
Alcohol consumption	1,131 (41.5)	990 (42.0)	141 (38.1)	0.156
Obese	804 (29.5)	690 (29.3)	114 (30.8)	0.550
BMI (kg/m^2)	23.5±3.1	23.5±3.1	23.6±3.2	0.736
Hypertension	1,014 (37.2)	864 (36.7)	150 (40.5)	0.152
Systolic BP (mmHg)	134±16	134±16	134±16	0.516
Diastolic BP (mmHg)	79±10	80±10	79±10	0.213
Diabetes	183 (6.7)	158 (6.7)	25 (6.8)	0.971
HbA1c (%)	5.6±0.6	5.6±0.6	5.6±0.6	0.645
FBG (mg/dL)	94.3±16.7	94.3±16.7	94.6±16.9	0.765
Hyperlipidemia	380 (13.9)	328 (13.9)	52 (14.1)	0.946
Total cholesterol (mg/dL)	201±32	201±31	203±33	0.341
Triglyceride (mg/dL)	105±64	106±64	105±63	0.855
HDL-C (mg/dL)	59±14	59±14	60±15	0.572
LDL-C (mg/dL)	125±30	124±30	126±30	0.369
eGFR (mL/min/1.73m^2)	81±16	82±16	80±16	0.170
Hemoglobin (mg/dL)	13.8±1.4	13.8±1.4	13.8±1.4	0.954
BNP (pg/mL)	18.7 [10.3–33.5]	18.6 [10.2–33.0]	19.2 [10.4–35.2]	0.258
GG/GT+TT in rs1808593 (n)	60/2,666	50/2,306	10/360	0.479

Unless indicated otherwise, data are expressed as the mean±SD, n (%), or median [interquartile range]. BMI, body mass index; BNP, B-type natriuretic peptide; BP, blood pressure; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

calculated by substituting the diet component in the renal disease equation with the Japanese coefficient.[12,16]

Total cholesterol was measured by the cholesterol oxidase enzyme method. Triglyceride was measured using an enzyme method. High-density lipoprotein cholesterol was measured using a chemically modified enzyme method. Fasting blood glucose (FBG) was measured using an enzyme electrode method. HbA1c levels were measured using HPLC.[15]

Definition of Cardiovascular Risks
Among the different cardiovascular risk factors, information regarding smoking history, alcohol consumption, history of heart failure, and family history of heart disease was collected from the health checkup questionnaire. Obesity was defined as a body mass index (BMI) ≥25 kg/m^2. Other definitions of cardiovascular disease risk in this study were as follows. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or the current use of antihypertensive medication. Diabetes was defined as FBG ≥126 mg/dL, HbA1c (National Glycohemoglobin Standardization Program) ≥6.5%, or the current use of antidiabetic medication. Hyperlipidemia was defined as total cholesterol ≥220 mg/dL, triglycerides ≥150 mg/dL, or the current use of antidyslipidemia medication.

Table 2. Comparisons of Clinical Characteristics Among rs1808593 Genotypes

Variables	GT/TT carriers (n=2,666)	GG carriers (n=60)	P value
Age (years)	63±10	61±10	0.179
No. males/females	1,209/1,457	23/37	0.280
Previous heart failure	97 (3.6)	1 (1.7)	0.417
Family history of CVD	454 (17.0)	12 (20.0)	0.546
Atrial fibrillation	36 (1.4)	1 (1.7)	0.834
Smoking	860 (32.3)	20 (33.3)	0.860
Alcohol consumption	1,111 (41.7)	20 (33.3)	0.195
Obese	788 (29.6)	16 (26.7)	0.627
BMI (kg/m²)	23.5±3.1	23.7±3.4	0.590
Hypertension	989 (37.1)	25 (41.7)	0.469
Systolic BP (mmHg)	134±16	136±16	0.527
Diastolic BP (mmHg)	79±10	80±11	0.488
Diabetes	181 (6.8)	2 (3.3)	0.290
HbA1c (%)	5.6±0.6	5.6±0.6	0.502
FBG (mg/dL)	94.3±16.8	95.2±14.3	0.684
Hyperlipidemia	368 (13.8)	12 (20.0)	0.171
Total cholesterol (mg/dL)	201±32	202±32	0.814
Triglyceride (mg/dL)	105±63	112±90	0.417
HDL-C (mg/dL)	59±14	63±17	0.051
LDL-C (mg/dL)	125±30	118±32	0.102
eGFR (mL/min/1.73 m²)	81±16	87±20	0.008
Hemoglobin (mg/dL)	13.8±1.4	13.7±1.3	0.657
BNP (pg/mL)	18.7 [10.3–33.3]	17.8 [8.8–39.0]	0.995
GG/GT+TT in rs1799983 (n)	2,306/360	50/10	0.479

Unless indicated otherwise, data are expressed as the mean±SD, n (%), or median [interquartile range]. Only eGFR differed significantly between the 2 groups. Abbreviations as in Table 1.

Statistical Analysis

All values are expressed as the mean±SD. Skewed values are presented as the median and interquartile range. Continuous variables were analyzed using Student's t-test or the Mann-Whitney U test. Categorical data were analyzed using the Chi-squared test. Cox proportional hazard analysis was used to determine independent predictors of future cardiovascular events. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Receiver operating characteristic (ROC) curves, the net reclassification index (NRI), and the integrated discrimination index (IDI) were calculated to determine the quality of improvement of the corrected reclassification following the addition of *NOS3* SNPs to the baseline model. Statistical significance was set at 2-tailed P<0.05. These statistical analyses were conducted using JMP version 14 (SAS Institute, Cary, NC, USA) and EZR (Saitama Medical Center, Jichi Medical University, Shimotsuke, Japan).[17] In addition, linkage disequilibrium analysis was performed using the R package "genetics".

Results

Baseline Characteristics and Clinical Characteristics Related to *NOS3* SNPs

The protocol used in this study is shown in **Figure 1**. Of the 2,726 subjects, 2,356 (86.4%) were homozygous for the G allele (GG), 356 (13.1%) were heterozygous for the G allele (GT), and 14 (0.5%) were homozygous for the T allele (TT)

of rs1799983. With regard to rs1808593, 60 (2.2%), 704 (25.8%), and 1,962 (72.0%) subjects were GG, GT, and TT carriers, respectively. SNP rs1799983 is located in exon 7 of the *NOS3* gene, whereas SNP rs1808593 is located in the intron 23 of the *NOS3* gene (**Figure 2**).[7,18] To examine the linkage disequilibrium of *NOS3* gene, linkage disequilibrium analysis was performed using data for these 2 SNPs. No significant association was found between these 2 SNPs (rs1799983, r^2=0.00006604155, Hardy-Weinberg equilibrium [HWE] P=0.679; rs1808593, r^2=0.0001281917, HWE P=0.610).
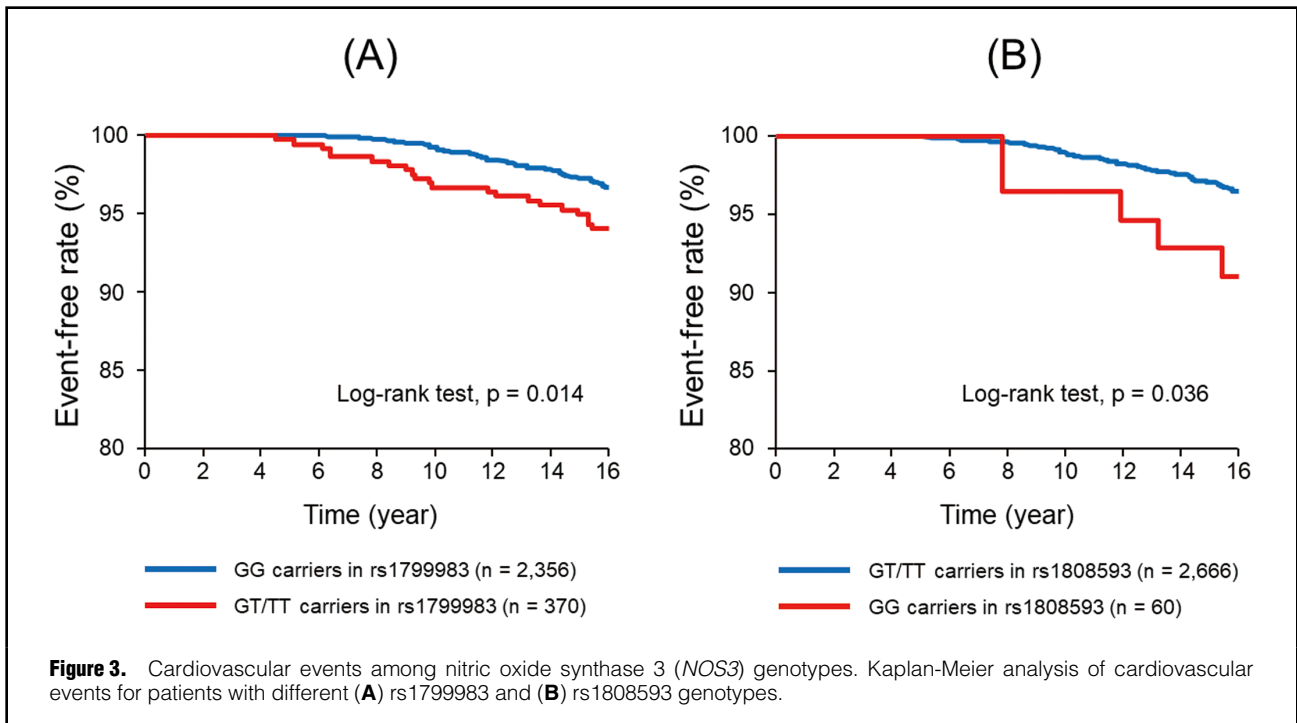
Subjects were divided into 2 groups based on the rs1799983 genotype, namely GG carriers (n=2,356) and GT/TT carriers (n=370). As indicated in **Table 1**, there were no significant differences in age, sex, the prevalence of previous heart failure, family history of cardiovascular disease, cardiovascular risk factors, and levels of eGFR, hemoglobin, and BNP between the 2 groups.

Subjects were also divided into 2 groups based on the rs1808593 genotype, namely GT/TT carriers (n=2,666) and GG carriers (n=60). As indicated in **Table 2**, there were no significant differences between the parameters, except for eGFR, between the 2 groups.

There was no significant association in allele frequency between the rs1799983 and rs1808593 genotypes (**Tables 1,2**).

Cardiovascular Events and *NOS3* SNPs

During the follow-up period, there were 96 cardiovascular events: 71 cardiovascular deaths and 25 non-fatal myocardial infarctions. Kaplan-Meier analysis demonstrated that

Figure 3. Cardiovascular events among nitric oxide synthase 3 (*NOS3*) genotypes. Kaplan-Meier analysis of cardiovascular events for patients with different (**A**) rs1799983 and (**B**) rs1808593 genotypes.

rs1799983 GT/TT carriers had a higher risk of developing cardiovascular events than rs1799983 GG carriers (**Figure 3A**). Kaplan-Meier analysis also demonstrated that rs1808593 GG carriers had a higher risk of developing cardiovascular events than rs1808593 GT/TT carriers (**Figure 3B**).

To examine the effects of *NOS3* SNPs more precisely, we also performed Kaplan-Meier analyses for cardiovascular death and myocardial infarction. Kaplan-Meier analysis demonstrated that those with the rs1799983 GT/TT genotype had a higher risk of cardiovascular death than those with the rs1799983 GG genotype. Kaplan-Meier analysis also demonstrated that those with the rs1808593 GG genotype had a higher risk of developing myocardial infarction than those with the rs1808593 GT/TT genotype (**Supplementary Figures 1,2**).

As shown in **Figure 4A**, univariate Cox proportional hazard regression analysis demonstrated that both rs1799983 GT/TT and rs1808593 GG were associated with cardiovascular events in the general population (GT/TT vs. GG carriers of rs1799983, hazard ratio [HR] 1.815, 95% confidence interval [CI] 1.119–2.945, P=0.016; GG vs. GT/TT carriers of rs1808593, HR 2.533, 95% CI 1.029–6.231, P=0.043). Multivariate Cox proportional hazard regression analysis demonstrated that both rs1799983 GT/TT and rs1808593 GG were significantly associated with cardiovascular events in the general population after adjusting for age, sex, smoking, obesity, hypertension, diabetes, hyperlipidemia, and eGFR (GT/TT vs. GG carriers of rs1799983, HR 1.813, 95% CI 1.110–2.961, P=0.018; GG vs. GT/TT carriers of rs1808593, HR 3.744, 95% CI 1.510–9.283, P=0.004; **Figure 4B**).

To examine whether the combination of *NOS3* SNPs could identify subjects at high risk of cardiovascular events in the general population, all subjects were divided into 3 groups based on the number of risk alleles in rs1799983 and rs1808593. Univariate Cox proportional hazard

regression analysis demonstrated that the number of risk alleles in the *NOS3* SNPs was related to cardiovascular events in the general population (**Figure 4C**). Multivariate Cox proportional hazard regression analysis demonstrated that the rate of cardiovascular events increased with an increasing number of risk alleles in *NOS3* SNPs in the general population, after adjusting for age, sex, smoking, obesity, hypertension, diabetes, hyperlipidemia, and eGFR (**Figure 4D**).
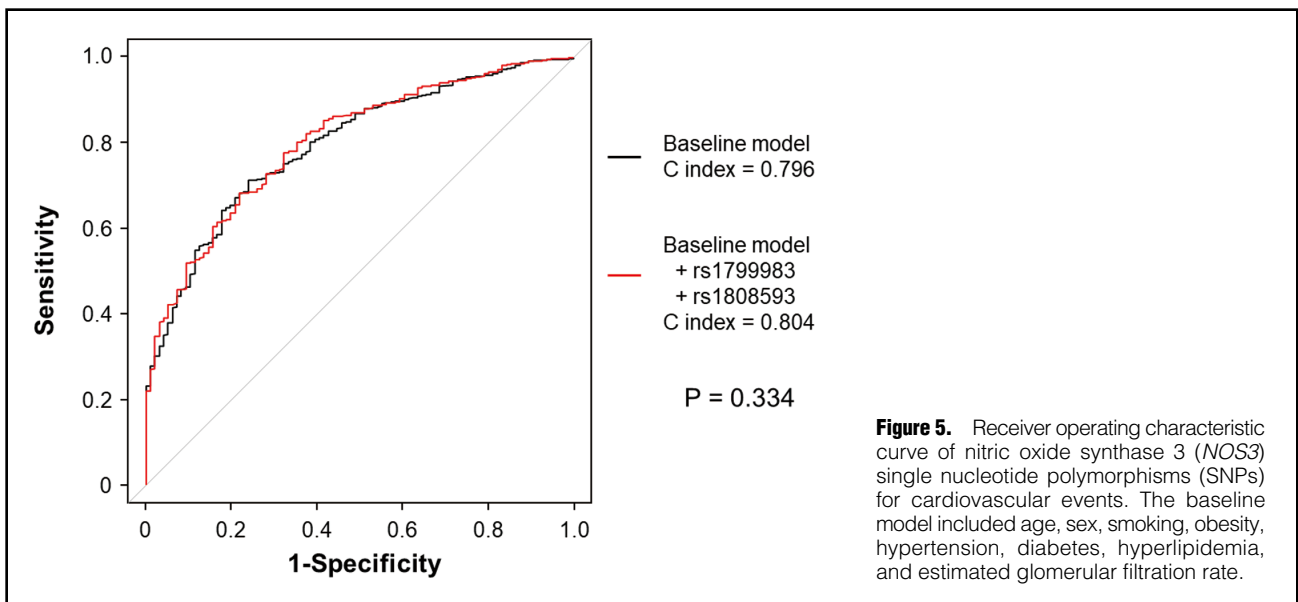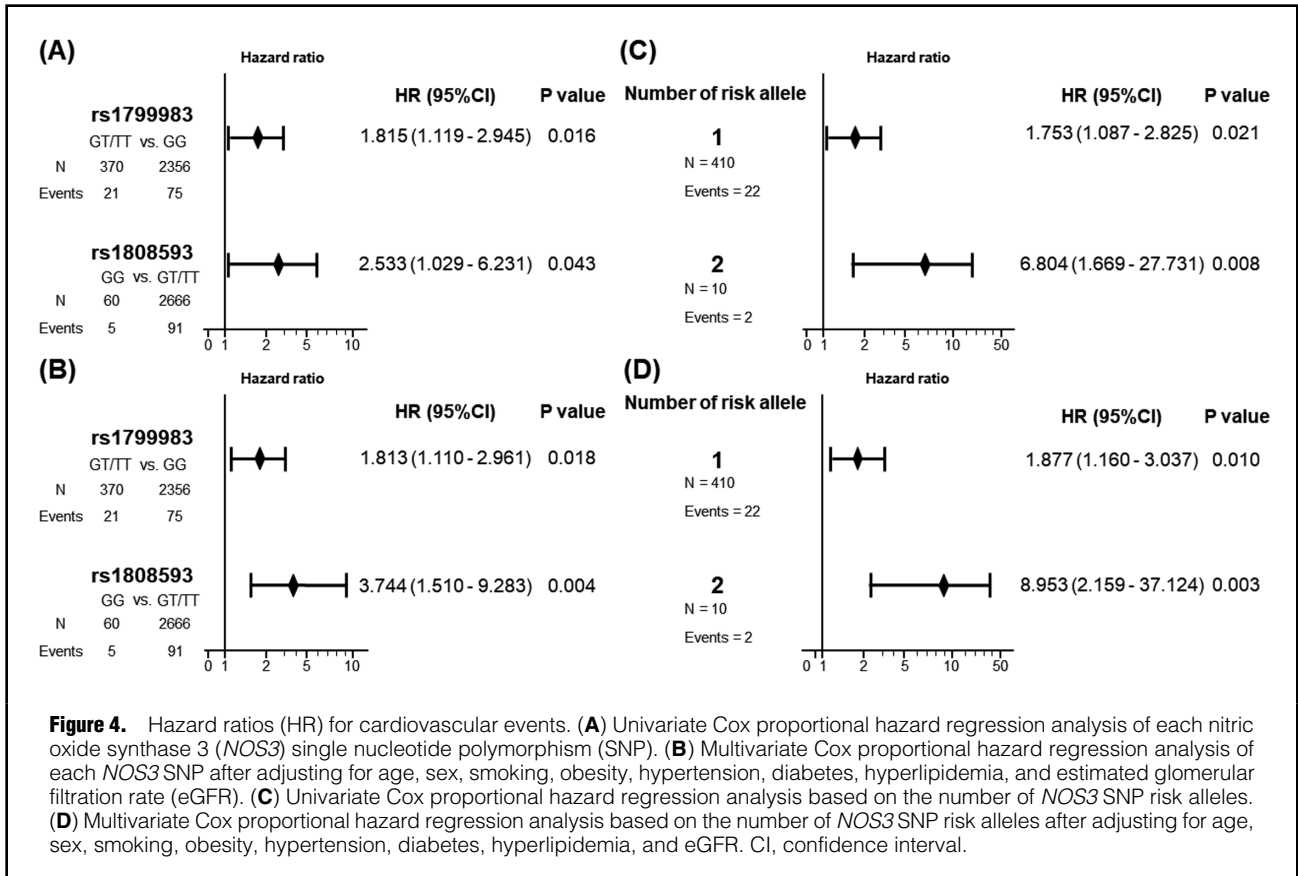
Improvement of Prognostic Value by Adding *NOS3* SNPs to the Baseline Model

To investigate whether the prediction model improved after adding the *NOS3* SNPs to the baseline model, improvements in the C index, NRI, and IDI were evaluated. The baseline model included age, sex, smoking, obesity, hypertension, diabetes, hyperlipidemia, and eGFR. The addition of rs1799983 and rs1808593 to the baseline model did not significantly improve the C index (0.796 vs. 0.804; P=0.334; **Figure 5**). However, NRI and IDI were significantly improved after the addition of the *NOS3* SNPs (**Table 3**).

Discussion

Main Findings

This study revealed that subjects in the general population with the rs1799983 GT/TT allele of the *NOS3* gene were at higher risk of cardiovascular events than those with the GG allele. Subjects in the general population with the rs1808593 GG allele of the *NOS3* gene were also at higher risk of cardiovascular events than those with the GT/TT allele. These associations remained significant after the multivariate models were adjusted for known risk factors. The prognostic value for predicting cardiovascular events in the general Japanese population was improved after the addition of *NOS3* SNPs.

Figure 4. Hazard ratios (HR) for cardiovascular events. (**A**) Univariate Cox proportional hazard regression analysis of each nitric oxide synthase 3 (*NOS3*) single nucleotide polymorphism (SNP). (**B**) Multivariate Cox proportional hazard regression analysis of each *NOS3* SNP after adjusting for age, sex, smoking, obesity, hypertension, diabetes, hyperlipidemia, and estimated glomerular filtration rate (eGFR). (**C**) Univariate Cox proportional hazard regression analysis based on the number of *NOS3* SNP risk alleles. (**D**) Multivariate Cox proportional hazard regression analysis based on the number of *NOS3* SNP risk alleles after adjusting for age, sex, smoking, obesity, hypertension, diabetes, hyperlipidemia, and eGFR. CI, confidence interval.

Figure 5. Receiver operating characteristic curve of nitric oxide synthase 3 (*NOS3*) single nucleotide polymorphisms (SNPs) for cardiovascular events. The baseline model included age, sex, smoking, obesity, hypertension, diabetes, hyperlipidemia, and estimated glomerular filtration rate.

Effect of Ethnicity on the Allele Frequency of *NOS3* SNPs

Previous studies have shown that the G allele in rs1799983 is a major genotype in Caucasian, Asian, and African American populations.[19,20] Similarly, the G allele in rs1799983 was found to be a major genotype in the present study. Conversely, previous studies have indicated a racial difference in the allele frequency of rs1808593: the G allele in rs1808593 is a major genotype in Caucasians,[10] but a minor genotype in the Chinese population.[21] In the present study, the G allele was a minor genotype in the general Japanese population, suggesting that the G allele in rs1808593 is a minor genotype in the Asian population.

Table 3. Statistics for Model Fit and Improvement With the Addition of *NOS3* SNPs (rs1799983 and rs1808593) on the Prediction of Cardiovascular Disease and Non-Fatal Myocardial Infarction

	C index	NRI (95% CI)	IDI (95% CI)
Baseline model	0.796	Reference	Reference
+*NOS3* SNPs	0.804 (P=0.334)	0.1990 (0.0235–0.3742; P=0.026)	0.0097 (0.0001–0.0194; P=0.049)

The baseline model included age, sex, smoking, obesity, hypertension, diabetes, hyperlipidemia and estimated glomerular filtration rate. CI, confidence interval; IDI, integrated discrimination index; *NOS3*, nitric oxide synthase 3; NRI, net reclassification index; SNPs, single nucleotide polymorphisms.

Cardiovascular Events and *NOS3* SNPs

To the best of our knowledge, this is the first prospective study to reveal that *NOS3* SNP polymorphisms (rs1799983 and rs1808593) are significantly associated with future cardiovascular events in the general population.

The SNP rs1799983, also known as Glu298Asp, substitutes guanine with thymine at position 894 of exon 7 of the *NOS3* gene, leading to a glutamine to aspartate change at position 298 of the protein.[7] The Glu298Asp polymorphism decreases NOS3 binding to caveolin-1 and reduces NOS3 bioavailability in caveolae.[7] This results in less NOS3 being available for calcium-activated calmodulin activation.[7] In addition, a meta-analysis demonstrated that the T allele in rs1799983 was associated with lower plasma NO concentrations.[22] Therefore, SNP rs1799983 modulates NOS3 activity and subsequent NO production. Several studies have demonstrated a close association between rs1799983 and cardiovascular diseases such as ischemic heart disease and cerebrovascular disease.[9,10,23] These observations support our hypothesis that, in the general Japanese population, the rs1799983 polymorphism is associated with future cardiovascular events through NO.

A previous study indicated no significant relationship between rs1808593 polymorphism and the presence of coronary artery disease in the Tunisian population.[24] However, it has been demonstrated that there is a close association between the rs1808593 polymorphism and peripheral artery disease, as indicated by the ankle-brachial index in patients with hypertension.[25] In the present study, the rs1808593 polymorphism was associated with future cardiovascular events independent of rs1799983. It has been reported that there is no significant association between rs1808593 and plasma NO concentrations in the Korean population.[26] Thus, a mechanism other than NO production could be contributing to the poor outcomes in the risk allele of rs1808593. However, no studies have examined the role of rs1808593 in NOS3 function. Because the present study was a prospective observational study, we could not determine the precise mechanism by which *NOS3* polymorphism worsened cardiovascular outcome in the general population. Thus, further studies are needed to understand the role of the rs1808593 SNP.

Importantly, risk alleles of both rs1799983 and rs1808593 were independently associated with cardiovascular events, and the combination of these SNPs identified subjects at high risk of cardiovascular events. The addition of *NOS3* SNP polymorphisms to established risk factors improved the capacity of models to predict cardiovascular events, indicating that information regarding *NOS3* SNP polymorphisms could be useful clinically for the prevention, early identification, and management of cardiovascular diseases. Therefore, this prospective study has expanded on past cross-sectional studies and reinforced that *NOS3* polymorphisms could be feasible markers for future cardiovascular events in the general Japanese population.

Study Limitations

This study has several limitations. First, the subjects in this study were local residents of Japan. Therefore, caution should be exercised when extrapolating the results of this study to the general population in other regions. Second, the baseline parameters in this study were measured only once during the community-based health checkup. Although the *NOS3* SNP polymorphism was reported to be associated with hypertension,[7] there was no significant difference in the baseline prevalence of hypertension among carriers of *NOS3* polymorphisms in the present study. This may be explained by the fact that this study included apparently healthy subjects, which differs from previous cross-sectional studies. Third, we did not measure NOS3 protein expression and activity or plasma NO concentrations. Therefore, it is unclear whether NO production was directly related to cardiovascular events in this study.

Conclusions

NOS3 gene polymorphisms could be a genetic risk factor for cardiovascular events in the general Japanese population, suggesting that they may facilitate the early identification of subjects at high risk of cardiovascular events.

Disclosures

None.

IRB Information

This study was approved by the Ethics Review Committee of Yamagata University Faculty of Medicine (No. 2020-414).

Data Availability

The deidentified participant data will not be shared.

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Supplementary Files

Please find supplementary file(s);
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