Common Genetic Variants on Bone Morphogenetic Protein Receptor Type IB (BMPR1B) Gene Are Predictive for Carotid Intima-Media Thickness

Yih-Jer Wu, MD, PhD; Yi-Nan Lee, PhD; Tzu-Wei Wu, PhD; Chao-Liang Chou, MD; Li-Yu Wang, PhD

Background: Bone morphogenetic proteins (BMP) 2 and 4 are implicated in the development of atherosclerosis. However, the relationships between the proteins, their main receptors and carotid intima-media thickness (cIMT), a predictive preclinical phenotype of atherosclerosis, have not been established.

Methods and Results: We screened and validated the relationships of single-nucleotide polymorphisms (SNPs) on BMP2, BMP4, BMPR1A, BMPR1B, and BMPR2 with thicker cIMT by 2 independent case-control studies that used different subject selection methods. Among 200 screened SNPs, 12 on BMPR1B were regarded as candidate genetic markers (P-value <5.0 ×10−4). After combining the discovery and validation studies and adjusting for traditional cardiovascular risk factors, rs4456963*G, rs4235438*T, rs2522530*T, and rs3796433*C showed significant higher odds ratios (ORs) of having thicker cIMT (adjusted ORs: 1.50–1.56; all P-values <2.5×10−4). Multivariate analyses showed that rs4456963 and rs3796433 were significantly independent determinants of cIMT thickening. The corresponding multivariate-adjusted ORs for rs4456963*G and rs3796433*C alleles were 1.50 (95% confidence interval (CI): 1.22–1.84) and 1.50 (95% CI: 1.23–1.82), respectively. Interaction between rs4456963 and rs3796433 was evident by the significantly higher OR (8.16, 95% CI: 3.12–21.3) for subjects with the CC genotype. The rs4456963*G and rs3796433*C showed positively linear trends with severity of carotid atherosclerosis.

Conclusions: We identified 2 SNPs on BMPR1B showing significantly independent correlations with thicker cIMT. The study provides invaluable evidence supporting that BMPR1B is closely related to carotid atherosclerosis and a potential target for the development of therapeutic agents for atherosclerotic disease.

Key Words: Atherosclerosis; Bone morphogenetic proteins; Bone morphogenetic protein receptors; Common carotid intima-media thickness; Single-nucleotide polymorphism

Atherosclerosis, the main underlying cause of cardiovascular disease (CVD), is a chronic inflammatory disease occurring in the intima and media of the medium- and large-sized arteries. The progress of atherosclerosis is determined by the combined effects of different cytokines that stimulate hyperplasia of smooth muscle cells in the intima. The increased thickness of intima may eventually cause the occlusion of arterioles, leading to stroke. Several large community- or population-based prospective studies have correlated carotid intima-media thickness (cIMT) with major cardiovascular events. Currently, cIMT measurement is widely used to assess the vascular health status of an individual and thicker cIMT is regarded as an intermediate phenotype of atherosclerosis.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, were initially identified as important signaling molecules in bone formation. BMPs are emerging as critical components in the regulation of several functions in multiple organs, including the cardiovascular system. Of the BMPs, BMP2 and BMP4 are the best characterized and are involved in pro-inflammatory functions. BMPs, as the other members of the TGF-β superfamily, are able to induce downstream responses through binding with type I and type II cell surface receptors – BMPRs.
type II BMP receptor for BMP2 and BMP4 are BMPR2. Among several identified type I BMP receptors, ALK3 (BMPR1A) and ALK6 (BMPR1B) show higher binding preferences for BMP2 and BMP4.6,7

Carotid IMT thickening is regarded as an early marker of atherosclerosis. A significant proportion of cIMT variations can be explained by genetic factors.11–13 BMP2 and BMP4 are correlated with the development and progression of atherosclerosis,14–18 so it is reasonable to hypothesize that genetic variants of BMP2, BMP4, and their receptor genes may also contribute to cIMT thickening. Therefore, we used 2 case-control studies to screen and validate the effects of common genetic variants of BMP2, BMP4, BMPR2, BMPR1A, and BMPR1B genes on cIMT thickening.

**Methods**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Study Subjects**

The study subjects of the discovery study were selected from participants in a previous community-based study.19 Of the 1,607 participants, 1,539 had good-quality recorded carotid ultrasound images and had never been diagnosed with CVD.20 We randomly selected a sample of 284 subjects who had a mean common carotid artery (CCA) IMT ≥ 0.70 mm, the 75th percentile of the distribution of the mean far-wall CCA IMT, as the cases, and randomly selected a sample of 464 subjects who had a mean CCA IMT < 0.70 mm as the controls.

The subjects of the validation study were selected from an ongoing community-based cohort of enrolled residents aged 40–74 years.21 During May 2014 to December 2016, 1,164 of 1,198 voluntarily participants had good-quality recorded carotid ultrasound images and had no CVD history. We selected a random sample of 282 subjects from those who had thicker cIMT (CCA IMT ≥ 0.70 mm) as the case group. Based on the age (40–49, 50–59, ≥ 60 years) and sex distribution of the case group, we performed stratified random sampling and selected a total of 282 subjects who had normal cIMT as the controls.

All participants of the 2 community-based studies voluntarily provided informed consent. The studies complied with the 1975 Helsinki Declaration on Ethics in Medical Research and were reviewed and approved by the Institution Review Board of Mackay Memorial Hospital (14MMHIS075).

**Measurement of Anthropometric and Cardiovascular Profiles**

Measurement of anthropometric traits has been described previously.19 A structured questionnaire was used to collect data of personal histories of common diseases and medicines, and health behaviors. We used an autoanalyzer (Toshiba TBA c16000; Toshiba Medical System, Holliston, MA, USA) with commercial kits (Denka Seiken, Tokyo, Japan) to determine the levels of total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting plasma glucose (FPG). In this study, hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg, diastolic BP (DBP) ≥ 90 mmHg, or a history of taking antihypertensives. Diabetes mellitus (DM) was defined as FPG ≥ 126 mg/dL or the use of hypoglycemic agents. Cigarette smoking was defined as having smoked cigarette for at least 4 days per week for ≥ 3 months.21

**cIMT Measurement**

We followed the protocol recommended by the American Society of Echocardiography22 to scan and obtain the ultrasound images of the CCA.23 In short, 2 experienced technicians operated high-resolution B-mode ultrasonography systems (GE Healthcare Vivid 7 and Vivid E9; General Electric Company, Milwaukee, USA), equipped with a multifrequency linear array transducer, to obtain the ultrasound images of both left and right CCAs. The far-wall average IMT of the distal 1–2 cm of the left and right CCAs was measure by another well-trained technician using automatic contouring software (GE Healthcare EchoPAC version 112.0.2; General Electric-Vingmed, Horten, Norway). All 3 technicians were blind to examinees’ clinical characteristics. In the study, mean cIMT used for statistical analyses was calculated as the mean of the left and right average IMT.

**Determination of Severity of Carotid Atherosclerosis**

The severity of carotid atherosclerosis was determined by 3 indicators, comprising the total number of carotid plaques, the maximum degree of carotid stenosis, and the carotid plaque score, as described previously.24 Carotid stenosis was defined as the percentage of maximal diameter reduction of the carotid arteries and calculated according to the European Carotid Surgery Trial criteria.24 The carotid plaque score took the total number of carotid plaques and degree of carotid stenosis into consideration and was constructed by summing the grades of the CCA bifurcation and the bilateral internal and external carotid arteries.25 An ordinal scale was assigned to carotid segments with differing severity of atherosclerosis: no observable plaque (grade=0), 1 small plaque with diameter stenosis < 30% (grade=1), 1 medium plaque with 30–49% diameter stenosis or multiple small plaques (grade=2), 1 large plaque with 50–99% diameter stenosis or multiple plaques with at least 1 medium plaque (grade=3), and 1 large plaque with 100% occlusion (grade=4).26

**SNP Selection**

In the discovery study, we used a plate to screen the associations of common genetic variants of the BMPs and BMPRs genes with cIMT. In the plate, there were 210 SNPs within 25-Kb up- or downstream of BMP2 (n=43), BMP4 (n=26), BMPR1A (n=41), BMPR1B2 (n=91), and BMPR2 (n=9). The eligibility of SNPs for association analyses had a call rate > 95%, a P-value of Hardy-Weinberg equilibrium (HWE) test in the controls > 0.001, and a minor allele frequency > 3%. The significance level of the discovery study was calculated as 0.05 divided by the number of eligible SNP, designed as uncorrected. To reduce the influence of overadjustment, we used 2×uncorrected as the preset critical value of candidate genetic markers. SNPs with a P-value less than the preset critical value were subjected to a validation study.

In addition to the candidate SNPs, genetic variants that were highly linked with candidate SNPs and may have influenced the expression and regulation of the associated BMPs and BMPRs were considered for the validation study. The linkage disequilibrium (LD) data in the 1000 Human Genome Project Phase 3-Southern Han Chinese26

Circulation Journal Vol.83, April 2019
were retrieved by using the Ensemble Genome Browser. The cutoff LD ($r^2$) value was set at 0.80.

Genotyping

The Axiom® CHB 1 Array Plate (Affymetrix Ltd, Santa Clara, CA, USA) and the Sequenom iPLEX MassARRAY system (Sequenom, San Diego, CA, USA) were used to determine the genotypes of subjects of the discovery and validation studies, respectively. All genotyping was performed by the National Center for Genome Medicine, Academica Sinica, Taiwan.

Statistical Analysis

In the study, we used Student’s t-test and the chi-square test to compare whether there were significant differences in the anthropometric and laboratory measurements between cases and controls. The relationships between thicker cIMT and common genetic variants of BMPs and BMPRs were assessed by the additive genotypic effect model. Assessment of pairwise LD of candidate SNPs were performed by Haploview 4.2 software.

Anthropometric and clinical factors significantly correlated with cIMT were subjected to multivariate analyses. Adjusted odds ratio (OR) and 95% confidence interval (CI) were calculated by logistic regression models. The Breslow-Day test was used to test the homogeneity of the estimated ORs of having thicker cIMT for the genetic markers between the discovery and validation studies. When the test statistic showed that the estimated ORs were not different between studies, the pooled ORs were obtained by summation of the study-specific ORs weighted by the inverse of their variances. To obtain the added predictabilities of genetic markers, the area under the receiver-operating characteristic curve (AUROC) was calculated for the basic and full models. The basic model contained all significant traditional cardiovascular risk factors. The full model contained all significant traditional cardiovascular risk factors and genetic markers. The Wald Chi-square test was used to evaluate the significance of increase in the AUROC by adding genetic markers to the basic model. We performed generalized linear model analyses to test the significance of associations between genotypes and severity of carotid atherosclerosis. All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Clinical Characteristics of Study Subjects

In the discovery study, the case group had significantly higher means of BMI, SBP, DBP, circumferences of the waist and hip, waist-to-hip ratio, total cholesterol, LDL-C, and cIMT than the control group. The mean level of LDL-C was significantly lower in the case group than in the control group (Table 1). As compared with the controls, the case group had a higher proportion of male sex and higher prevalence rates of hypertension and DM. In the validation study, we observed significant differences in BMI, waist and hip circumferences, BPs, LDL-C, HDL-C, and cIMT between cases and controls.

Among 210 screened SNPs, 10 were excluded from association analysis because of an inadequate call rate (n=5), a minor allele frequency <3.0% (n=3), and a P-value of the HWE test <1×10^{-3} (n=2; Supplementary Table 1). Among the 200 eligible SNPs for association analyses, 12 located on BMPR1B had a P-value less than the preset value 2×αcorrection (2×0.05/200=5.0×10^{-5}) and were regarded as candidate genetic markers (Table 2). None of the other screened SNPs on BMP2, BMP4, BMPR1A, and BMPR2 was eligible for the validation study. The minor allele frequencies of the 10 candidate SNPs were significantly higher in the case group than in the control group, with ORs of having thicker cIMT ranging from 1.42 to 1.64. In contrast, rs2522530*C and rs3796433*A alleles were more frequent in the control group than in the case group.

### Table 1. Clinical Characteristics of Subjects of the Discovery and Validation Studies*

| Variable                      | Discovery study | Validation study |
|-------------------------------|-----------------|------------------|
|                               | Cases (n=284)   | Controls (n=464) | P value |
|                               |                 |                  |
| Male sex, n (%)               | 157 (55.3)      | 213 (45.9)       | 0.013   |
| Age at enrollment (years)     | 58.9 (8.8)      | 52.6 (8.7)       | <0.001  |
| Cigarette smoking, n (%)      | 52 (18.6)       | 74 (16.1)        | 0.40    |
| BMI (kg/m²)                   | 25.9 (3.3)      | 24.1 (3.5)       | <0.001  |
| Waist circumference (cm)      | 84.9 (8.4)      | 80.1 (8.9)       | <0.001  |
| Hip circumference (cm)        | 96.5 (6.2)      | 94.2 (9.2)       | <0.001  |
| Waist-to-hip ratio (%)        | 88.0 (6.4)      | 84.9 (7.4)       | <0.001  |
| SBP (mmHg)                    | 135.0 (17.7)    | 126.1 (19.5)     | <0.001  |
| DBP (mmHg)                    | 82.6 (13.4)     | 78.6 (13.3)      | <0.001  |
| Hypertension, n (%)           | 134 (47.4)      | 134 (28.9)       | <0.001  |
| Total cholesterol (mg/dL)     | 216.2 (42.6)    | 205.7 (36.0)     | <0.001  |
| LDL-C (mg/dL)                 | 133.8 (35.8)    | 121.4 (33.2)     | <0.001  |
| HDL-C (mg/dL)                 | 52.0 (14.4)     | 56.9 (16.0)      | <0.001  |
| FPG (mg/dL)                   | 108.7 (36.0)    | 97.1 (25.9)      | <0.001  |
| Diabetes mellitus, n (%)      | 39 (13.7)       | 25 (8.4)         | <0.001  |
| cIMT (mm)                     | 0.799 (0.091)   | 0.599 (0.058)    | <0.001  |

*Values in the table are mean (SD), unless specified. BMI, body mass index; cIMT, carotid intima-media thickness; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.
In the validation study, the genotype distributions of all, except rs6849425 and rs3796417, candidate SNPs were significantly different between groups (Table 2). There were significant trends with the ORs of having thicker cIMT for all, except rs1444929*A, minor alleles. The trends were significantly inverse for rs2522530*C and rs3796433*A alleles and were significantly positive for the other 6 minor alleles.

It is considered appropriate to pool the results of the discovery and validation studies (Table 3). After combining them and adjusting for traditional cardiovascular risk factors, rs4456963*G, rs4235438*T, rs2522530*T, and

### Table 2. Association Analyses for 12 Candidate SNPs on BMPR1B With Thicker cIMT

| SNP          | Risk allele | Allele A/B* | Cases     | Controls   | PGT** | Additive effects (OR† (95% CI)) | PRT†† |
|--------------|-------------|-------------|-----------|------------|-------|---------------------------------|-------|
| rs1553743    | G/T         | 21.7        | 19/85/180 | 16.3       | 5/141/318 | 1.2×10⁻⁴ | 1.43 (1.09–1.87) | 8.7×10⁻³ |
| rs7694987    | G/A         | 23.1        | 18/95/171 | 15.7       | 4/138/322 | 2.8×10⁻⁵ | 1.64 (1.25–2.15) | 3.2×10⁻⁴ |
| rs4456963    | G/A         | 24.3        | 21/96/167 | 17.1       | 8/143/313 | 1.9×10⁻⁴ | 1.56 (1.20–2.03) | 7.3×10⁻⁴ |
| rs4235438    | T/A         | 27.6        | 29/99/156 | 20.3       | 15/158/291 | 2.7×10⁻⁴ | 1.49 (1.17–1.90) | 1.2×10⁻³ |
| rs2522530    | C/T         | 24.1        | 14/109/161| 30.0       | 32/202/210 | 1.1×10⁻³ | 0.64 (0.51–0.82) | 2.8×10⁻⁴ |
| rs3775007    | G/A         | 25.5        | 27/91/166 | 19.1       | 11/155/298 | 8.7×10⁻⁵ | 1.44 (1.13–1.85) | 3.6×10⁻³ |
| rs6532515    | A/G         | 37.3        | 51/110/123| 29.2       | 37/197/230 | 2.1×10⁻⁴ | 1.42 (1.14–1.76) | 1.6×10⁻³ |
| rs1444929    | A/G         | 32.7        | 42/102/140| 27.2       | 29/194/241 | 4.7×10⁻⁴ | 1.29 (1.03–1.62) | 2.4×10⁻² |
| rs6849425    | T/C         | 20.8        | 19/80/185 | 15.2       | 4/133/327 | 1.4×10⁻⁵ | 1.46 (1.12–1.92) | 5.8×10⁻³ |
| rs3796417    | T/C         | 20.8        | 20/78/186 | 15.2       | 5/131/328 | 5.9×10⁻⁵ | 1.45 (1.11–1.90) | 6.2×10⁻² |
| rs3796433    | A/C         | 20.6        | 13/91/180 | 30.3       | 49/183/232 | 3.4×10⁻⁴ | 0.61 (0.48–0.78) | 6.6×10⁻⁵ |

*Allele A/B, minor/major alleles. **PGT: P value for the Chi-square test of genotype distribution between groups. †OR of having thicker cIMT for the minor alleles. ††PTR: P value for the additive genotypic model. cIMT, carotid intima-media thickness; OR, odds ratio; SNP, single-nucleotide polymorphism.

### Table 3. Combined Analyses for the Minor Alleles of 9 Candidate SNPs on BMPR1B With Thicker cIMT

| SNP          | Risk allele | Discovery study | Validation study | Combined study |
|--------------|-------------|----------------|-----------------|---------------|
| rs1553743    | G           | 1.34 (0.99–1.80) | 0.059 | 1.65 (1.20–2.27) | 0.0022 | 0.38 | 1.47 (1.18–1.83) | 5.1×10⁻⁴ |
| rs4456963    | G           | 1.48 (1.10–1.98) | 0.0087 | 1.66 (1.23–2.25) | 9.8×10⁻⁴ | 0.60 | 1.56 (1.27–1.93) | 2.9×10⁻⁵ |
| rs4235438    | T           | 1.38 (1.05–1.80) | 0.021 | 1.64 (1.24–2.18) | 5.1×10⁻⁴ | 0.83 | 1.50 (1.23–1.82) | 4.8×10⁻⁵ |
| rs2522530    | T           | 1.69 (1.30–2.02) | 0.0001 | 1.39 (1.05–1.82) | 0.021 | 0.80 | 1.54 (1.27–1.85) | 1.2×10⁻⁵ |
| rs3775007    | G           | 1.40 (1.06–1.85) | 0.019 | 1.47 (1.11–1.95) | 0.0081 | 0.50 | 1.43 (1.17–1.75) | 5.1×10⁻⁴ |
| rs1444929    | A           | 1.30 (1.02–1.68) | 0.038 | 1.17 (0.89–1.53) | 0.27 | 0.66 | 1.24 (1.03–1.49) | 0.023 |
| rs6849425    | T           | 1.47 (1.09–2.00) | 0.013 | 1.35 (0.99–1.85) | 0.060 | 0.86 | 1.41 (1.14–1.76) | 1.9×10⁻³ |
| rs3796417    | T           | 1.48 (1.10–2.00) | 0.010 | 1.40 (1.03–1.91) | 0.032 | 0.27 | 1.44 (1.16–1.79) | 8.3×10⁻⁴ |
| rs3796433    | C           | 1.79 (1.37–2.38) | 2.3×10⁻⁵ | 1.32 (1.00–1.72) | 0.046 | 0.14 | 1.54 (1.27–1.85) | 1.1×10⁻⁵ |

*ORs adjusted for age, sex, SBP, BMI, LDL-C, and cigarette smoking. **PBD: P-value of the Breslow-Day test. Abbreviations as in tables 1,2.
BMPs and Carotid IMT

rs3796433*C*C showed significant associations with thicker cIMT (P-value <2.5×10^{-4}). The corresponding pooled adjusted ORs were 1.56 (95% CI=1.27–1.93), 1.00 (95% CI=1.23–1.82), 1.54 (95% CI=1.27–1.85), and 1.54 (95% CI=1.27–1.85), respectively. The pooled adjusted ORs for rs1553743*G, rs3775007*G, and 3796417*T were non-significantly increased (P-values: 5.1×10^{-4} –1.0×10^{-3}).

The results of multivariate analyses showed that only rs4456963 and rs3796433 remained significantly associated with cIMT (Table 4; Model I). The multivariate-adjusted ORs of having thicker cIMT were 1.50 (95% CI: 1.22–1.84) and 1.50 (95% CI: 1.23–1.82) for rs4456963*G and rs3796433*C, respectively. Model II showed that as compared with the rs4456963 AA genotype, the multivariate-adjusted ORs were 1.36 (95% CI: 1.05–1.76) and 2.95 (95% CI: 1.60–5.44) for the AG and GG genotypes, respectively. The multivariate-adjusted ORs were 2.06 (95% CI: 1.25–3.38) and 2.76 (95% CI: 1.70–4.48) for the rs3796433 AC and CC genotypes, respectively, in the comparison with the rs3796433 AA genotype. Model III showed that as compared with the rs4456963 AA-rs3796433 AA combinatorial genotype, the adjusted ORs of having thicker cIMT were elevated for all, except the AG-AA genotype, other combinatorial genotypes and was the highest for the GG-CC genotype (adjusted OR=8.16; 95% CI: 3.12–21.33). There was no significant difference in the adjusted ORs of having thicker cIMT between male and female subjects (Supplementary Table 3).

The AUROC for the basic model that contained all significant traditional cardiovascular risk factors, including age, sex, SBP, BMI, LDL-C, and cigarette smoking, was 0.7015 (95% CI=0.6737–0.7293) and were 0.7201–0.7224 for Models I–III. The Wald Chi-square test showed that the addition of 2 genetic markers to the basic model significantly improved the discrimination (all P-values of χ² test <0.005).

The rs4456963*G and rs3796433*C alleles were both significantly positively correlated with higher means of cIMT (Table 5). rs4456963*G was also positively correlated with higher means of waist-to-hip ratio, LDL-C, and FPG. The means of the total number of carotid plaques, maximum stenosis, and carotid plaque score were the lowest for the rs4456963 AA genotype and were the highest for the rs4456963 GG genotype, yet the trends had borderline

### Table 4. Multivariate Logistic Regression Analyses for Thicker cIMT*

| Genetic marker | OR** (95% CI) | P value |
|---------------|--------------|---------|
| Model I       |              |         |
| rs1553743*G   | NS           |         |
| rs4456963*G   | 1.50 (1.22–1.84) | 1.47×10^{-4} |
| rs4235483*T   | NS           |         |
| rs2522530*C   | NS           |         |
| rs3775007*G   | NS           |         |
| rs1444929*A   | NS           |         |
| rs6849429*T   | NS           |         |
| rs3496417*T   | NS           |         |
| rs3796433*C   | 1.50 (1.23–1.82) | 2.58×10^{-4} |
| AUROC         | 0.7201 (0.6930–0.7472) |         |
| Model II      |              |         |
| rs4456963     |              |         |
| AA            | 1.00         |         |
| AG            | 1.36 (1.05–1.76) | 0.019 |
| GG            | 2.95 (1.60–5.44) | 5.39×10^{-4} |
| rs3796433     |              |         |
| AA            | 1.00         |         |
| AC            | 2.06 (1.25–3.38) | 4.48×10^{-3} |
| CC            | 2.76 (1.70–4.48) | 4.21×10^{-6} |
| AUROC         | 0.7217 (0.6947–0.7487) |         |
| Model III     |              |         |
| rs4456963     | rs3796433    |         |
| AA            | AA           | 1.00    |
| AA            | AC           | 1.83 (1.02–3.28) | 0.044 |
| AA            | CC           | 2.46 (1.39–4.37) | 2.00×10^{-3} |
| AG            | AA           | 0.82 (0.28–2.41) | 0.71  |
| AG            | AC           | 2.69 (1.42–5.09) | 2.46×10^{-3} |
| AG            | CC           | 3.37 (1.85–6.13) | 6.88×10^{-5} |
| GG            | AC           | 4.48 (1.56–12.89) | 5.43×10^{-3} |
| GG            | CC           | 8.16 (3.12–21.33) | 1.87×10^{-5} |
| AUROC         | 0.7224 (0.6954–0.7493) |         |

*Subjects of discovery and validation studies. **ORs adjusted for age, sex, SBP, BMI, LDL-C, and cigarette smoking. AUROC, area under the receiver-operating characteristic curve; NS, not significant. Other abbreviations as in tables 1, 2.
rs3796433*C was not correlated with any traditional cardiovascular risk factors. There were significant trends for rs3796433*C with the total number of carotid plaques or carotid plaque score. The trend with maximum stenosis had borderline significance.

**Discussion**

TGF-β signaling is crucial for the maintenance of vascular homeostasis and dysregulation of this pathway has been reported to be proatherogenic. Although the role of TGF-β in atherosclerosis is well studied, the roles of other members of the superfamily and their receptors are yet to be clearly established. In the study, we first demonstrate that common genetic variants of BMPR1B, a receptor for many TGF-β superfamily members, were significantly correlated with thicker cIMT, which is a robust indicator of future atherosclerotic events.

In the study, we first screened 200 SNPs on BMP2, BMP4, BMPRIA, BMPRIB, and BMPR2 for their relationships with thicker cIMT using a case-control study that randomly selected subjects from our previous community-based study. We identified 12 promising SNPs on BMPRIB and further validated their relationship with thicker cIMT using a frequency-matched case-control study that selected subjects from our ongoing community-based study. After combining subjects of the discovery and validation studies and adjusting for traditional cardiovascular risk factors, we found 4 SNPs (rs1553743, rs4456963, rs4235438, and rs3796433) showing significant correlations with the likelihood of having thicker cIMT. Multivariate analyses showed that rs4456963 and rs3796433 were significantly independent determinants of cIMT thickening and correlated with the severity of carotid atherosclerosis. The addition of these 2 genetic markers to the basic model, which included all significant traditional cardiovascular risk factors, significantly improved predictability and discrimination. Furthermore, interaction of these 2 significant SNPs was evident by the significantly higher risk for subjects who had the rs4456963 GG-rs3796433 CC genotype. More importantly, the rs4456963*G and rs3796433*C alleles were common in the study population, as well as in other ethnic groups, so our findings have significant implications for cardiovascular medicine and public health. TGF-β signaling is crucial for physiological processes, including embryonic development, angiogenesis and wound healing. The pleiotropic effects of TGF-β signaling can be either proatherogenic or antiatherogenic in the progress of CVD. TGF-β superfamily induces downstream signaling through the binding of cell surface receptors. BMPR1B is the receptor for many TGF-β superfamily ligands, some of which have been shown to regulate the functions of vascular endothelial cells and smooth muscle cells. It is reasonable to hypothesize that the inconsistent findings of the effects of the TGF-β superfamily on the cardiovascular system are attributable to variations in the regulation of BMPR1B expression. The former hypothesis is supported by studies of pulmonary artery hypertension (PAH), a disease characterized by arteriole hyperplasia. In pulmonary arterial smooth muscle cells isolated from a sporadic PAH patient bearing no mutation in BMPR2, BMPR1B influenced mitosis as well as intracellular BMP signaling. A recent genetic study screened for mutations in endoglin, SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, SMAD6, SMAD7, BMPRIA and BMPRIB genes in 43 idiopathic PAH patients who had no mutations in BMPR2, ALK1 and SMAD9. Two missense mutations in BMPRIB were each identified in 2 patients and correlated with the activities of SMAD4 and SMAD8. Taken together, alteration of BMPR1B function may potentially affect atherogenesis and atherosclerotic progression. As far as we know, however, there has not been a report demonstrating the relationship between BMPR1B and atherosclerosis.

In humans, BMPRIB locates at 4q22.3 encoding 253 nucleotides of 5’-UTR, 13 exons, and 3,774 nucleotides of 3’-UTR. The minor allele frequencies of exonic variants of BMPRIB are very rare, indicating that it plays important roles in homeostasis. In addition to PAH, BMPRIB confers susceptibility to several human diseases, including brachydactylies, cataracts and endometriosis. The pathogenesis of these diseases is closely related to the rs1434536 polymorphism in the 3’-UTR of BMPRIB. rs1434536 locates in the direct target site of micro RNA-125b that differentially regulates the C and T alleles of rs1434536.
No report has correlated polymorphisms in the 3’-UTR of BMPR1B with atherosclerosis or cardiovascular risk factors.

In contrast to the findings in carcinogenesis, the present study showed that the 3’-UTR polymorphisms of BMPR1B were unlikely to be the primary pathogenesis of carotid atherosclerosis. The Taiwan Biobank shows that among subjects who have whole-genome sequence data (n=1,517), 10 SNPs in the 3’-UTR have a minor allele frequency >3.0%.42 The LD data of the Southern Han Chinese shows that these 3’-UTR SNPs can be grouped into 3 LD blocks, designed A, B, and C.26 Block A includes rs1434536, rs1863564, rs1434535, and rs2162450. Block B includes rs1836261, rs11097457, and rs2289044, and Block C includes rs7023107 and rs7389218.26 In the discovery study, several eligible SNPs, which had high LD with these LD blocks, including rs7756042, rs3796438, rs7662630, and rs7023055, or members of the block (rs1836261) were not correlated with cIMT (Supplementary Table 1). To confirm our findings, we selected 2 SNPs from each LD block and assayed the genotypes of study subjects in the validation study (primers for PCR reaction and annealing for the 6 3’-UTR SNPs are shown in Supplementary Table 2). The results of the association analyses showed that none of these 3’-UTR SNPs was significantly correlated with thicker cIMT (Supplementary Table 4).

Except for the 3’-UTR polymorphisms, the minor allele frequencies of genetic variants locating in the 5’-UTR, exons, and regulatory sites as well as the non-coding transcripts of BMPR1B, are all less than 2.0% in Taiwanese,42 as well as in other ethnic populations.26 In this study, 6 SNPs up- and downstream of BMPR1B were also included and showed no significant association with cIMT. However, the screened SNPs are only part of the SNPs up- and downstream of BMPR1B. Accordingly, the regulation of transcription of BMPR1B may be through differential binding of regulators with polymorphic sites on the introns and down- and upstream regions of the gene. It is also possible that the efficiency of splicing among these intronic variants may vary. Further investigation is necessary to clarify our speculations.

We also found that both rs4456963*G and rs3796433*C were linearly correlated with the severity of carotid atherosclerosis. Additionally, rs4456963*G also correlated with higher means of waist-to-hip ratio, LDL-C, and FPG, but rs3796433*C was not correlated with any traditional cardiovascular risk factors. These findings indicated that rs4456963*G and rs3796433*C may possibly correlate with different pathogenic pathways of atherosclerosis. The hypothesis needs further exploration.

There are potential limitations of the present study. Although we did not identify common variants of BMP2, BMP4, BMPR1A, and BMPR2 showing significant correlation with thicker cIMT, the negative finding does not necessarily preclude the possibility that they may play important roles in atherothrombotic diseases. In the study, the numbers of SNPs selected for each gene varied (e.g., 92 SNPs on BMPR1B and 9 SNPs on BMPR2). Accordingly, some causative SNPs may not have been included and some screened genes seem more likely to suffer from false-negative bias in the study. In addition, to control the overall type I error and to reduce the likelihood of false-negative findings at the same time, only SNPs with a P-value less than 2-folds of the significance level were included in the validation study. Even still, false-negative and false-positive findings may still exist.

In conclusion, the study identified 2 SNPs on BMPR1B showing significantly independent correlations with cIMT thickening. The study provides invaluable evidence supporting that BMPR1B is closely related to atherosclerosis and a potential target for the development of therapeutic agents for atherosclerotic diseases. Importantly, the rs4456963*G and rs3796433*C alleles were common in the study population as well as in other ethnic groups. These 2 novel SNPs may serve as useful biomarkers in addition to traditional cardiovascular risk factors in the prediction of future atherosclerosis, and thus, promote early health intervention in high-risk people.

Conflict of Interest Statement

The authors declare no conflict of interest.

Author Contributions

Y.-J.W. and L.-Y.W. contributed to the study conception and design; C.-C.L. and W.-L.Y. acquired data; W.-L.Y. analyzed the data; Y.-J.W. and Y.-N.L. prepared the paper; L.-Y.W. supervised the study. All authors critically revised the manuscript. All authors read and approved the final manuscript.

Name of Grant

This work was supported by the Ministry of Science and Technology of Taiwan (grant no. MOST 104-2314-B-715-002-MY3), Mackay Medical College (1061B10), and the Wang Jhan-Yang Public Charitable Trust Fund (WJY 2017-HR-01).

References

1. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317–325.
2. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: The Rotterdam Study. Circulation 1997; 96: 1432–1437.
3. Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklro M, Sharrett AR, et al. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: The Atherosclerosis Risk in Communities (ARIC) Study, 1987–1993. Am J Epidemiol 1997; 146: 483–494.
4. O’Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults: Cardiovascular Health Study Collaborative Research Group. N Engl J Med 1999; 340: 14–22.
5. Gordon KJ, Blobe GC. Role of transforming growth factor-beta superfamily signaling pathways in human disease. Biochim Biophys Acta 2008; 1782: 197–228.
6. Lowery JW, de Caestecker MP. BMP signaling in vascular development and disease. Cytokine Growth Factor Rev 2010; 21: 287–298.
7. Cai J, Pardali E, Sánchez-Duffhues G, ten Dijke P. BMP signaling in vascular diseases. FEBS Lett 2012; 586: 1993–2002.
8. Helbing T, Rothweler R, Ketterer E, Goetz L, Heinke J, et al. BMP2-induced inflammation can be suppressed by the proinflammatory phenotype of endothelium. Blood 2011; 118: 5040–5049.
9. Shen J, James AW, Zara JN, Asarian G, Khadarian K, Zhang JB, et al. BMP2-induced inflammation can be suppressed by the osteoinductive growth factor NELL-1. Tissue Eng Part A 2013; 19: 2390–2401.
10. Sanders LN, Schoenhard JA, Saleh MA, Mukherjee A, Ryzhou S, McMaster WG Jr, et al. BMP antagonist gremlin 2 limits inflammation after myocardial infarction. Circ Res 2016; 119: 434–449.
11. Lee K, Sung J, Lee SC, Park SW, Kim YS, Lee JY, et al. Segment-specific carotid intima-media thickness and cardiovascular risk factors in Koreans: The Healthy Twin Study. Eur J Prev Cardiol 2012; 19: 1161–1172.
12. Zhao J, Cheema FA, Bremner JD, Goldberg J, Su S, Sniadz, et al. Heritability of carotid intima-media thickness: A twin
study. *Atherosclerosis* 2008; 197: 814 – 820.

13. Medda E, Fagnani C, Schillaci G, Tarnoki AD, Tarnoki DL, Baracchini C, et al. Heritability of arterial stiffness and carotid intima-media thickness: An Italian twin study. *Nutr Metab Cardiovasc Dis* 2014; 24: 511 – 517.

14. Boström K, Watson KE, Horn S, Wortham C, Herman IM, Derer LL. Bone morphogenetic protein expression in human atherosclerotic lesions. *J Clin Invest* 1993; 91: 1800 – 1809.

15. Nakagawa Y, Ikeda K, Akakabe Y, Koide M, Uraoka M, Yutaka KT, et al. Paracrine osteogenic signals via bone morphogenetic protein-2 accelerate the atherosclerotic intimal calcification in vivo. *Arterioscler Thromb Vasc Biol* 2010; 30: 1908 – 1915.

16. Finkenzeller G, Hager S, Stark GB. Effects of bone morphogenetic protein 2 on human umbilical vein endothelial cells. *Microvasc Res* 2012; 84: 81 – 85.

17. Derwall M, Malhotra R, Lai CS, Beppu Y, Aitkawa E, Seehra JS, et al. Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2012; 32: 613 – 622.

18. Feng J, Gao J, Li Y, Yang Y, Dang L, Ye Y, et al. BMP4 enhances foam cell formation by BMPR-2/Smad1/5/8 signaling. *Int J Mol Sci* 2014; 15: 5536 – 5552.

19. Wu TW, Chan HL, Hung CL, Lu JJ, Wang SD, Wang SW, et al. Differential patterns of effects of age and sex on metabolic syndrome in Taiwan: Implication for the inadequate internal consistency of the current criteria. *Diabetes Res Clin Pract* 2014; 105: 239 – 244.

20. Wu TW, Hung CL, Liu CC, Wu YJ, Wang LY, Yeh HI. Associations of cardiovascular risk factors with carotid intima-media thickness in middle-age adults and elders. *J Atheroscler Thromb* 2017; 24: 677 – 686.

21. Wu TW, Chou CL, Chen YC, Juang YL, Wang LY. Associations of common genetic variants on IL-17 genes and carotid intima-media thickness. *J Atheroscler Thromb* 2018; 25: 1156 – 1167.

22. Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: A consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *J Am Soc Echocardiogr* 2008; 21: 93 – 111.

23. Chou CL, Wu YJ, Hung CL, Liu CC, Wang SD, Wu TW, et al. Segment-specific prevalence of carotid artery plaque and stenosis in middle-aged adults and elders in Taiwan: A community-based study. *J Formos Med Assoc* 2018; 117: 64 – 71.

24. Rothwell PM, Warlow CP. Low risk of ischemic stroke in patients with reduced internal carotid artery lumen diameter due to low poststenotic flow? On behalf of the European Carotid Surgery Trialists’ Collaborative Group. *Stroke* 2000; 31: 622 – 630.

25. Chien KL, Su TC, Jeng JS, Hsu HC, Chang WT, Chen MF, et al. Carotid artery intima-media thickness, carotid plaque and coronary heart disease and stroke in Chinese. *PLoS One* 2008; 3: e3435.

26. Yates A, Akanni W, Amode MR, Barrett D, Billis K, Carvalho-Silva D, et al. Ensembl 2016. *Nucleic Acids Res* 2016; 44: D710 – D716.

27. Ensemble Genome Browser 86. http://asia.ensembl.org/Homo_sapiens/Info/Index (accessed December 10, 2016).

28. Barrett JC, Fry B, Maller J, Daly MJ. Haplview: Analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; 21: 263 – 265.

29. Wolf YG, Rasmussen LM, Ruoslahti E. Antibodies against transforming growth factor-beta 1 suppress intimal hyperplasia in a rat model. *J Clin Invest* 1994; 93: 1172 – 1178.

30. Schullick AH, Taylor AJ, Zhu W, Qui CB, Dong G, Woodward RN, et al. Overexpression of transforming growth factor beta in arterial endothelium causes hyperplasia, apoptosis, and cartilaginous metaplasia. *Proc Natl Acad Sci USA* 1998; 95: 6983 – 6988.

31. Grainger DJ, Kemp PR, Metcalfe IC, Liu AC, Lawn RM, Williams NR, et al. The serum concentration of active transforming growth factor-beta is severely depressed in advanced atherosclerosis. *Nat Med* 1995; 1: 74 – 79.

32. Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK. Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *J Clin Invest* 2003; 112: 1342 – 1350.

33. Pardali E, Ten Dijke P. TGF-β signaling and cardiovascular diseases. *Int J Biol Sci* 2012; 8: 195 – 213.

34. Dyer LA, Pi X, Patterson C. The role of BMPs in endothelial cell function and dysfunction. *Trends Endocrinol Metab* 2014; 25: 472 – 480.

35. Takeda M, Otsuka F, Nakamura K, Inagaki K, Suzuki J, Miura D, et al. Characterization of the bone morphogenetic protein (BMP) system in human pulmonary arterial smooth muscle cells isolated from a sporadic case of primary pulmonary hypertension: Roles of BMP type IB receptor (activin receptor-like kinase-6) in the mitotic action. *Endocrinology* 2004; 145: 4344 – 4354.

36. Chida A, Shintani M, Nakayama T, Furutani Y, Hayama E, Inai K, et al. Missense mutations of the BMPR1B (ALK6) gene in childhood idiopathic pulmonary arterial hypertension. *Circ J* 2012; 76: 1501 – 1508.

37. Mundlos S. The brachydactylies: A molecular disease family. *Clin Genet* 2009; 76: 123 – 136.

38. Khan S, Basit S, Khan MA, Muhammad N, Ahmad W. Genetics of human isolated acromesomelic dysplasia. *Eur J Med Genet* 2014; 57: 123 – 136.

39. Khan S, Basit S, Khan MA, Ahmad W, Genetics of human isolated acromesomelic dysplasia. *Eur J Med Genet* 2014; 57: 123 – 136.

40. Chang CY, Chen Y, Lai MT, Chang HW, Cheng J, Chan C, et al. A risk variant in an miR-125b binding site in BMPR1B is associated with breast cancer pathogenesis. *Cancer Res* 2009; 69: 7459 – 7465.

41. Chang CY, Chen Y, Lai MT, Chang HW, Cheng J, Chan C, et al. BMPR1B up-regulation via a miRNA binding site variation defines endometriosis susceptibility and CA125 levels. *PLoS One* 2013; 8: e60630.

42. Feng N, Xu B, Tao J, Li P, Cheng G, Min Z, et al. A miR-125b binding site polymorphism in bone morphogenetic protein membrane receptor type IB gene and prostate cancer risk in China. *Mol Biol Rep* 2012; 39: 369 – 373.

43. Taiwan Biobank. https://taiwanview.twbiobank.org.tw/index (accessed August 16, 2017).

**Supplementary Files**

Please find supplementary file(s): http://dx.doi.org/10.1253/cirrq.CJ-18-1046