BRAP Activates Inflammatory Cascades and Increases the Risk for Carotid Atherosclerosis

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The BRCA-1 associated protein gene (BRAP) was recently identified as a susceptibility gene for myocardial infarction (MI). In the present study we aimed to decipher the association between the BRAP polymorphism and carotid atherosclerosis and the mechanism underlying its proatherogenic effect. A total of 1749 stroke/MI-free volunteers received carotid ultrasonic examinations for the measurement of intima-medial thickness (IMT) and plaque. The promoter polymorphism rs11066001 was selected because it affects the transcription of BRAP. We found that the GG genotype was associated with a 1.58-fold increased risk for having at least one plaque compared to carrying the A allele (P = 0.021). When subjects were divided by the cutoff value of IMT above the mean plus 1 standard deviation, there was an overrepresentation of the GG genotype in the subjects with thicker IMT (P = 0.004). The expression of BRAP increased significantly when human aortic smooth muscle cells (HASMCs) were treated with lipopolysaccharide (LPS). HASMCs were transfected with small interfering RNA against BRAP or scrambled sequences before treatment with LPS. Knockdown of BRAP led to attenuated HASMC proliferation and reduced secretion of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) in response to LPS. Downregulation of BRAP did not affect the protein levels of nuclear factor-κB (NF-κB), but prohibited its nuclear translocation. Coimmunoprecipitation experiments confirmed an interaction between BRAP and the two major components of the IKK signalosome, IKKβ and IKKγ. Collectively, BRAP conferred a risk for carotid plaque and IMT. Inflammatory stimuli upregulated BRAP expression, and BRAP activated inflammatory cascades by regulating NF-κB nuclear translocation.

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INTRODUCTION

Ultrasound measurements of intima-media thickness (IMT) and plaque at the carotid arteries are predictors of future cardiovascular risk (1,2). Each 1-standard deviation (SD) increment in IMT leads to a 1.26-fold increased risk for myocardial infarction (MI) (3), and the presence of plaque is associated with a 4.1-fold risk (4). Although both phenotypes correlate well with pathologically and clinically defined atherosclerosis, they represent distinct traits with unique relationships to atherosclerosis (5). IMT is akin to a physical effect of adapting to aging and hypertensive stress, whereas plaque corresponds to a more pathogenic alteration in the vessel walls (6). These two phenotypes are used as independent surrogate markers in genetic studies of cardiovascular diseases.

The BRCA-1 associated protein (BRAP) gene was recently found to be a susceptibility gene for MI (7), in which the polymorphism rs11066001 in this gene was significantly associated with MI risk in Japanese and Taiwanese populations (7). This finding was further replicated by an independent study in Japanese and Korean populations (8). In addition, rs1106601 was found to confer a risk for lower ankle-brachial index in the Taiwanese population (9). Because atherosclerosis is the common pathogenesis shared by MI and peripheral artery disease, these association studies high-
lighted the effect of BRAP on the atherogenic process. BRAP was originally identified as a cytoplasmic protein that inhibited the nuclear translocation of the breast cancer suppressor protein BRCA-1 (10). It modulated the differentiation of monocytes through binding to the nuclear localization signal (NLS) of the cell-cycle inhibitor p21 (11). BRAP is also known to impede mitogen signaling propagation (IMP), the threshold modulator of the Raf–mitogen-activated protein kinase–extracellular signal regulated kinase (Raf-MEK-ERK) pathway (12). By repressing the Raf-MEK-ERK signal cascades, BRAP influences the secretion of interferon-γ in CD4 T cells (13). However, its role in the cardiovascular system remains to be explored.

Lines of evidence support the fact that atherosclerosis is a chronic inflammatory disease (14). Atherosogenesis is initiated by accumulation of lipid components in the vessel walls. Recruitment of leukocytes and secretion of inflammatory cytokines subsequently open a vicious cycle of inflammatory response, leading to plaque formation and further thrombotic events (14). Many genes involved in the different stages of atherosclerosis are regulated by the transcription factor nuclear factor-κB (NF-κB) (15). For example, monocyte chemoattractant protein-1 (MCP-1), the crucial chemokine for monocyte recruitment, is activated by NF-κB (16). Genes encoding vascular adhesion molecules, matrix metalloproteinases (MMPs), and interleukins are all downstream targets of NF-κB. Genetic polymorphisms at the MCP-1 (17), MMP-3 (18,19), MMP-9 (20) and interleukin-6 (IL-6) (21) genes were found to be associated with carotid atherosclerosis. In our previous study (7), BRAP was found to be involved in the NF-κB–dependent inflammatory pathway. Therefore, genetic variants in BRAP may also contribute to the disease susceptibility of carotid atherosclerosis. The aims of the present study were to test for the influence of BRAP polymorphism on carotid atherosclerosis and to elucidate the mechanism underlying the BRAP proatherogenic effect. We first tested the association between BRAP polymorphism and carotid atherosclerosis in human subjects. The single-nucleotide polymorphism (SNP) rs11066001 was selected because it affects the transcription of BRAP and has shown the strongest association with MI risks (7). In the second part of this study, we conducted in vitro experiments to clarify how BRAP influenced the activation of NF-κB and promoted the atherosclerotic process.

**Materials and Methods**

**Study Subjects**

Stroke- and MI-free volunteers were enrolled from Kaohsiung Medical University Hospital (KMUH) from 2006 to 2009. The investigation conformed to the principles outlined in the Declaration of Helsinki. All study protocols and methods were approved by the local institutional review board of KMUH. Demographic data and histories of hypertension, diabetes mellitus, hypercholesterolemia and cigarette smoking were obtained from each subject. Body height and weight were measured for the calculation of body mass index (BMI). All subjects received carotid ultrasonic examinations for the measurement of carotid plaque and IMT. Among the 1749 subjects, 316 had only plaque data and 1433 had data on both phenotypes. Venous blood was collected for biochemical analyses and genomic DNA extraction. The SNP rs11066001 (270A>G at intron 3) was genotyped by using the TaqMan genotyping assay (Applied Biosystems, Foster City, CA, USA). Briefly, polymerase chain reaction (PCR) primers and two allele-specific probes were designed to detect the specific SNP target. The PCR reactions were performed in 96-well microplates with an ABI 7500 real-time PCR machine (Applied Biosystems). Allele discrimination was achieved by detection of fluorescence by use of ABI 7500 System SDS software version 1.2.3 (Applied Biosystems). The genotype calling rate was 96.1%.

**Cell Culture and Small Interfering RNA Transfection**

Primary HASMCs (Cascade Biologics, Portland, OR, USA) were grown in culture medium containing medium 231, smooth muscle cell growth supplement, fetal bovine serum (10%), amphotericin B (50 ng/mL) and gentamycin (50 μg/mL) (both purchased from Cascade Biologics). The cells were incubated at 37°C in 95% air/5% CO₂ atmosphere. Passages 4 to 9 were used for experiments. After adherence, cells were starved in serum-free media for 24 h to induce quiescence. Cells were then incubated with lipopolysaccharide (LPS) (Escherichia coli O111:B4 1μg/mL; Sigma-Aldrich, St. Louis, MO, USA), oxidized LDL (ox-LDL 40 μg/mL; Biomedical Technologies, Stoughton, MA, USA) or simvastatin (10 μmol/L;
Sigma-Aldrich) for different periods of time as indicated.

Gene silencing was performed 24 h prior to the addition of LPS. Control small interfering RNA (siRNA) targeting scrambled sequences (12935-200), siRNA against BRAP (BRAP-HSS112138) and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Cells were transfected with 50 pmol control siRNA or siRNA against BRAP per 10^6 cells by using Lipofectamine 2000.

### Measurements of HASMC Proliferation and Migration, and MCP-1/Interleukin-8 Concentration

To measure HASMC proliferation, microplates were incubated at 37°C for 24 h. After that, 0.5 mg/mL of dimethyl-thiazol-diphenyltetrazoliumbromide (Sigma-Aldrich) was added into each well and the cells were incubated for 2.5 h at 37°C. Spectrophotometric readings were done by use of an X340 spectrophotometer at 595 nm (BioTek Instruments, Winooski, VT, USA). Cell migration ability was evaluated by using a transwell assay (Millipore, Billerica, MA, USA). HASMCs were evaluated by using a transwell assay (Millipore, Billerica, MA, USA). HASMCs were transfected with LPS 1 μg/mL for 60 min, the cells were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100 (GIBCO-BRL, Grand Island, NY, USA). Slides were washed with phosphate buffered saline (PBS) and incubated in blocking buffer (1% bovine serum albumin in PBS; GIBCO-BRL). The cells were then incubated with anti-NF-κB p65 autoantibody (1:100; Santa Cruz Biotechnology) overnight at 4°C. Slides were later incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti–rabbit autoantibody (1:1000; Invitrogen), and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI 1:100; Invitrogen). Fluorescence was detected with a Leica DMRE confocal microscope equipped with an argon laser source (Leica, Mannheim, Germany).

### S-Tag Pull-Down Assay

An S-tagged BRAP expression plasmid was constructed by using a pTriEx-4 vector (Novagen, Darmstadt, Germany). Human embryonic kidney 293 (HEK293) cells (obtained from the Health Science Research Resources Bank, Osaka, Japan; JCRB9068) in 150-mm dishes were transiently transfected with BRAP-pTriEx-4 or pTriEx-4 vector. The cells were lysed and diluted 10-fold by using S-protein bind/wash buffer. The extracts were incubated with an S-protein agarose for 12–18 h at 4°C. The agarose was washed 3× in S-protein bind/wash buffer and one time in Tris/HCl buffer (10 mmol/L Tris, pH 8.0, with 150 mmol/L NaCl). Bound S-tagged proteins were eluted by glycine/HCl buffer (100 mmol/L glycine, 0.5 mol/L NaCl, pH 2.7) and neutralized by 1 mol/L Tris/HCl (pH 9.0). The protein complexes were concentrated and analyzed by SDS-PAGE and stained by using a silver stain MS kit (Wako Chemicals, Mountain View, CA, USA). The protein bands were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI/TOF) mass spectrome-
try at Shimadzu TechnoResearch, Kyoto, Japan.

Coimmunoprecipitation Experiments
We conducted coimmunoprecipitation (Co-IP) experiments to test the interactions between BRAP and three components of the IκK-signalosome, which were (a) the inhibitor of kappa light polypeptide gene enhancer in B-cell, kinase beta (IKBKB, aka IKKβ), (b) the nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha (NFKBIA, aka IkBa), and (c) the nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, beta (NFKBIB, aka IkBβ). Expression plasmids of Myc- or S-tagged BRAP or IKBKB were transfected into COS7 cells (HSRRB; JCRB9127) by using Fugene6 (Roche, Mannheim, Germany). Immunoprecipitation was performed in lysis buffer, which contained 20 mmol/L Tris, pH 7.5, with 150 mmol/L NaCl, 0.4% Nonidet P-40, 5 μg/mL of proteasome inhibitor MG-132 and a protease inhibitor tablet without ethylenediaminetetraacetic acid (Roche). Twenty-four h after transfection, cells were lysed and immunoprecipitation was done by using an anti-Myc tag (Santa Cruz Biotechnology) on S-protein agarose (Novagen). We visualized the immune complex by using HRP-conjugated S protein (Novagen) or anti-Myc antibody peroxidase conjugates (Santa Cruz Biotechnology).

COS7 cells were again transfected with expression plasmids of FLAG-tagged or S-tagged BRAP, NFKBIA or NFKBIB. Cells were lysed and immunoprecipitation was done using an anti-FLAG tag (Santa Cruz Biotechnology) on S-protein agarose. The immune complex was visu-

Table 1. Demographic data of the study participants (N = 1749).

| Phenotype                      | Genotype | Age, mean ± SD, y | Sex, M | Hypertension | Diabetes | Hypercholesterolemia | Past and current smoker | Total cholesterol, mean ± SD, mg/dL | Triglyceride, mean ± SD, mg/dL | HDL-cholesterol, mean ± SD, mg/dL | Body mass index, mean ± SD, Kg/m² | CCA IMT value, mean ± SD, mm | Bif IMT value, mean ± SD, mm | ICA IMT value, mean ± SD, mm | Presence/absence of plaque, n (%) | Failed genotyping |
|-------------------------------|----------|------------------|--------|-------------|----------|----------------------|-------------------------|-------------------------------------|----------------------------------|-----------------------------------|---------------------------------|-----------------|-----------------|-----------------|--------------------------|------------------|
| Presence                      |          | 55.2 ± 10.6 (19-87) | 41.1%  | 34.4%       | 12.1%    | 33.8%                | 18.6%                   | 200.9 ± 38.1                      | 122.3 ± 31.4                      | 56.5 ± 15.2                      | 24.5 ± 3.5                      | 0.63 ± 0.14                    | 0.66 ± 0.13                    | 0.51 ± 0.09                   | 583 (33.3%)/1166 (66.7%) | 68 (3.9%) |
| BRAP SNP rs11066001           |          | AA               |        |             |          |                      |                         | 841 (48.1%)                       | 707 (40.4%)                       | 133 (7.6%)                       |                                |                               |                               |                               |                          |

Table 2. The association between SNP rs11066001 in BRAP and carotid atherosclerosis.

| Phenotype                      | Genotype | Statistics (recessive model) |
|-------------------------------|----------|------------------------------|
| Carotid plaque                 |          |                              |
| Absence                        |          |                              |
| AA                             | 563 (50.6%) | Logistic regression<sup>a</sup> |
| AG                             | 472 (42.4%) | Reference                    |
| GG                             | 77 (6.9%)  |                              |
| Presence                       |          |                              |
| AA (n = 700)                   |          |                              |
| AG (n = 571)                   |          |                              |
| GG (n = 100)                   |          |                              |
| CCA mean ± SD, mm              |          | Multivariate regression<sup>a</sup> |
| Bif mean ± SD, mm              |          |                              |
| ICA mean ± SD, mm              |          |                              |
| Dichotomized IMT, ≥ mean + 1 SD versus < mean + 1 SD | Logistic regression<sup>a</sup> |
| CCA                            |          |                              |
| versus 52.0%                   | 46.0%     | OR = 2.25 (1.30–3.89)        |
| versus 41.4%                   | 43.1%     | P = 0.004                    |
| versus 6.6%                    | 10.9%     |                              |
| Bif                            |          |                              |
| versus 51.5%                   | 49.0%     | OR = 1.12 (0.61–2.03)        |
| versus 41.2%                   | 43.7%     | P = 0.720                    |
| versus 7.3%                    | 7.3%      |                              |
| ICA                            |          |                              |
| versus 50.5%                   | 54.5%     | OR = 0.77 (0.40–1.50)        |
| versus 42.0%                   | 39.6%     | P = 0.445                    |
| versus 7.5%                    | 5.9%      |                              |

<sup>a</sup>P value obtained from multivariate regression analysis with adjustment for age, sex, hypertension, diabetes, hyperlipidemia, BMI and smoking. Only covariates with P < 0.05 were kept in the regression models.
alized by using HRP-conjugated S protein or anti-FLAG antibody peroxide conjugates (Santa Cruz Biotechnology).

Statistical Analysis

Genotype distributions were tested for Hardy-Weinberg equilibrium (HWE) by using the goodness-of-fit test. Logistic regression and $\chi^2$ squared tests were used to compare the genotype distributions between subjects with at least one plaque and those with no plaques. We analyzed both the continuous IMT data as well as the dichotomized IMT data. For the continuous IMT data, ANOVA and Student $t$ test were used to compare the mean IMT values across different genotypes. For the dichotomized IMT data, subjects with IMT values above the mean plus 1 SD were defined as high-risk individuals, and the rest of study subjects were defined as reference individuals. Logistic regression with adjustment for other cardiovascular risk factors (diabetes, hypertension, hypercholesterolemia, BMI and smoking) were used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the risk genotype. Only covariates with a $P$ value < 0.05 were kept in the regression model. Because we tested the BRAP effect on two phenotypes (that is, IMT and plaque), the Bonferroni-corrected $P$ value was adopted for multiple testing correction.

For the cellular experiments, variables were presented as mean ± SD. Student $t$ test was used to compare the variables between the treatment and control groups. All experiments were performed at least three times with technical duplicates in each sample.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org)

RESULTS

BRAP Genetic Polymorphism and Carotid Atherosclerosis

The demographic features of the study participants are shown in Table 1. The genotype distribution of rs11066001 was in HWE. We used two intermediate phenotypes (that is, carotid IMT and plaque) to assess the BRAP effect on atherosclerosis. When subjects with at least one plaque were compared with those without any plaque, the frequency of minor homozygote GG was overrepresented in the former (9.8% versus 6.9%, Table 2). Compared with the subjects with the AG or AA genotype, those carrying the GG genotype had a 1.58-fold greater risk for having at least one plaque (nominal $P = 0.021$; Bonferroni-corrected $P = 0.042$).

Continuous IMT data indicated that subjects carrying the GG genotype had a thicker IMT at CCA than those with the AA or AG genotype ($0.65 \pm 0.16$ mm versus $0.63 \pm 0.14$ mm respectively, Table 2). However, the association was not statistically significant (nominal $P = 0.089$). For the dichotomized IMT data, subjects with the GG genotype had an OR of 2.25 (nominal $P$ value = 0.004, Bonferroni-corrected $P$ value = 0.008) for a thicker CCA IMT in comparison to the A allele carriers. The average IMT values at Bif or ICA were not significantly different among individuals with AA, AG or GG genotype.

We further tested the associations between rs11066001 and other cardiovascular risk factors (that is, sex, hypertension, diabetes, hypercholesterolemia, smoking...
status and BMI). The genotype distribution did not show any significant difference between subjects with or without any of the risk factors (nominal P = 0.21–0.86, Supplementary Table 1).

**LPS Induced BRAP Upregulation and HASMC Proatherogenic Changes**

We conducted a series of in vitro experiments to elucidate the BRAP mechanism underlying atherosclerosis. LPS stimulation significantly increased the mRNA levels and protein amount of BRAP in HASMCs (Figures 1A, B), whereas the ox-LDL treatment did not cause any BRAP expression changes (Figure 1C). Augmented mRNA and protein levels of NFKB1 were observed along with the increase of BRAP expression (Figures 1A, B). Treating HASMCs with 10 μmol/L simvastatin prior to the LPS stimulation did not reverse the upregulation of BRAP expression (Figure 1D).

LPS enhanced the proliferation and migration of HASMCs (Supplementary Figures 1A, B). After the cells were treated with LPS, there was a time-dependent increment in cell proliferation and migration. The concentrations of MCP-1 and IL-8 in the culture medium also increased gradually after HASMCs were exposed to LPS (Supplementary Figures 1C, D).

**Knockdown of BRAP Attenuated the Proatherogenic Effect**

We then knocked down BRAP by siRNA to clarify the relationship between BRAP expression and the proatherogenic phenotypes observed in HASMCs. BRAP siRNA significantly suppressed BRAP mRNA levels and protein amounts (Figures 2A, B). When BRAP was downregulated, the secretion of inflammatory cytokines (MCP-1 and IL-8) was reduced in response to LPS stimulation (Figures 2C, D). Treating HASMCs with 10 μmol/L simvastatin prior to the LPS stimulation did not reverse the upregulation of BRAP expression (Figure 1D).

**BRAP Influenced the Nuclear Translocation of NF-κB**

We hypothesized that the proatherogenic effect of BRAP might be attributable to its influence on NF-κB. Knocking down BRAP did not influence NFKB1 mRNA levels (Figure 3A). However, the nuclear translocation of NF-κB p65 and p50 decreased markedly in cells transfected with BRAP siRNA (Figure 3B). When the HASMCs were treated with LPS, the nuclear/cytoplasm ratio of NF-κB protein increased gradually in a time-dependent manner (Figure 3C, Supplementary Table 2). On the contrary, there was no change in the nuclear/cytoplasm ratio of NF-κB protein in HASMCs with BRAP knockdown prior to LPS stimulation. Similar findings were also demonstrated by immunofluorescence stains (Supplementary Figure 3).

**BRAP Interacted with IKBKB and NFKBIB, but Not NFKBIA**

During identification of proteins that interact with BRAP by using an S-tag pull-down assay and MALDI/TOF mass analyses, we found IKBKB (aka IKKβ) as a possible binding partner of BRAP protein. To examine the interaction between BRAP protein and IKBKB, we constructed plasmids expressing Myc-tagged or S-tagged BRAP or IKBKB in COS7 cells. The Co-IP experiments confirmed their interaction in protein blot.
analysis (Figure 4A). We further transfected COS7 cells with plasmids containing FLAG-tagged or S-tagged BRAP, NFKBIA (aka IkBα) or NFKBIB (aka IkBβ). The protein blot analysis showed that BRAP interacted with NFKBIB, but not with NFKBIA (Figures 4B, C).

**BRAP Affected NFKBIB Degradation, but Not NFKBIA**

We then used Western blot to evaluate the influence of BRAP silencing on IkB degradation. For HASMCs transfected with control siRNA, the protein amount of IkBα and IkBβ decreased gradually after the cells were treated with LPS (Figure 3D, Supplementary Table 3). The LPS stimulation increased the phosphorylation of IkBα and IkBβ in a time-dependent manner, which was parallel to the degradation of IkBα and IkBβ (Figure 3D). When the HASMCs were transfected with BRAP siRNA, there was no significant change in the total amount of IkBβ or in the concentrations of phosphorylated IkBβ. This result suggested that BRAP silencing would attenuate the phosphorylation and degradation of IkBα in response to the LPS stimulation. On the other hand, BRAP silencing did not affect the LPS-induced degradation of IkBα (Figure 3D, Supplementary Table 3). There was a time-dependent decrement in the total IkBα amount in response to the LPS treatment if the HASMCs were transfected with BRAP siRNA or with control siRNA. These findings were coherent with the results from Co-IP experiments, in which BRAP protein interacted with IkBβ, but not with IkBα.

**DISCUSSION**

Carotid plaque and IMT are surrogate markers of cardiovascular diseases. The major pathways that are implicated in atherosclerosis include abnormal lipid metabolism, endothelial dysfunction, thrombosis/platelet aggregation and arterial inflammation (23). The results of the present study demonstrated that BRAP’s effect on cardiovascular diseases is primarily mediated by its proinflammatory activity. First, BRAP expression was induced by LPS but not ox-LDL. Second, knockdown of BRAP hampered LPS-induced cell proliferation and MCP-1/IL-8 secretion. In addition, reduced expression levels of BRAP inhibited NF-κB activation by reducing the nuclear translocation of NF-κB. We also demonstrated that BRAP interacted with two major components of the IKK-signalsome, NFKBIB and IKKBK. The NFKBIB (aka IkBβ) sequesters the NF-κB complex in the cytoplasm, and IKKBK (aka IKKβ) allows NF-κB to translocate into nucleus by initiating the degradation of IkBα (24,25). Western blot analysis further confirmed that BRAP silencing could influence the degradation of IkBβ. Taken together, LPS-induced BRAP upregulation activated the IKK-signalsome, enhanced NF-κB nuclear translocation and consequently increased the expression of inflammatory cytokines.

The present study is the first to demonstrate that BRAP is upregulated by an inflammatory stimulant, leading to the secretion of inflammatory cytokines. We are also the first to demonstrate that BRAP may influence NF-κB nuclear translocation, a prerequisite of NF-κB ac-
We found that BRAP directly binds to IκBβ and IKKβ, both of which are the major components of the IκK signalosome. IKKβ, the major subunit of IκB kinase, phosphorylates IκB in response to inflammatory cytokines, LPS and tumor necrosis factor-α (26). IKKβ-dependent phosphorylation subsequently triggers polyubiquitination and degradation of IκB. It exposes the NF-κB NLS masked by IκB and initiates the shuttle of NF-κB into the nucleus (27).

The transcription factor NF-κB controls the activation of various atherogenic genes like vascular cellular adhesion molecule-1 (VCAM-1), E-selectin and MMPs (24). By regulating the nuclear translocation of NF-κB, BRAP might influence other proatherogenic genes in addition to MCP-1 and IL-8 (16,28).

Notably, we found that BRAP bound to IκBβ but not IκBα. It has been found that IκBβ is degraded only when cells are stimulated by LPS and interleukin-1 (IL-1), whereas IκBα is degraded by all of the known inducers of NF-κB (24). IκBα replenishes quickly after NF-κB activation, resulting in a rapid but transient effect on cells (29). IκBβ, on the other hand, is not resynthesized immediately and causes a slow but long-term activation of NF-κB (29). Previous studies also revealed that unphosphorylated IκBβ may compete with IκBα and lead to persistent activation of NF-κB (25,30). The present findings indicate that BRAP’s effect on NF-κB translocation might be mediated through influencing the IKK signalosome. However, we do not know whether BRAP was involved in the phosphorylation, ubiquitination or proteolysis of IκBβ. In our data, BRAP knockdown did not change the NF-κB nuclear/cytoplasm ratio when the cells were not treated with LPS. Accordingly, BRAP by itself probably does not initiate the NF-κB translocation. BRAP protein may be a chaperone of the IKK signalosome and modulates the activation of NF-κB signal cascades. Further studies are warranted to clarify these possible functions of BRAP.

In the present study, the minor allele of rs11066001 in BRAP was associated with a thicker CCA IMT, as well as the presence of plaque. Our results are consistent with the detrimental effect of this minor allele observed in patients with MI (7). Because the minor allele G has greater transcription efficacy than the common allele A (7), individuals with the GG genotype may have higher levels of BRAP protein and thus manifest more severe atherogenic phenotypes. Statins, the widely used antiatherosclerotic drugs with antiinflammatory effects, did not reverse BRAP overexpression in response to LPS stimulation. A recent study showed that statins inhibited LPS-induced NF-κB activation via inhibiting the Rho/Rho kinase signal pathway (31). Because NF-κB activation could be regulated by various signal pathways, individuals of the GG genotype might be exposed to a higher risk of cardiovascular events despite receiving statin therapy.

We observed significant associations between SNP rs11066001, plaque and dichotomized IMT data. Although both IMT and plaque are predictors of cardiovascular risks, the two phenotypes measure different aspects of atherogenesis (5). IMT mainly indicates hypertensive hypertrophy of the vessel walls (6), whereas plaque reflects a later stage of
atherosclerosis in which lipid infiltration, inflammation, matrix overproduction, endothelium dysfunction and smooth muscle cell proliferation take place (32). In cellular experiments, knockdown of BRAP significantly reduced the secretion of inflammatory cytokines and had a modest effect on HASMC proliferation and a minor effect on the HASMC migration. Because we used the cutoff value of IMT above the mean plus 1 SD in analyzing IMT data, our results indicate that the effect of BRAP effect may be more evident when the atherosclerosis is advanced.

There were several limitations to the present study. We acknowledge that a significant threshold of nominal P < 0.05 may have led to false-positive results when several phenotypes were tested concurrently. We presented both the nominal P values and the Bonferroni-corrected P values. The significance threshold represented a trade-off between avoidance of false-positive associations while taking into account that a set of related phenotypes were tested in the present study. The validation of BRAP's effect in HASMC further strengthened the association observed in human subjects. We analyzed both continuous and dichotomized IMT data because a certain threshold may be needed to detect the atherogenic effect (2). We selected the mean plus 1 SD as the cutoff level to dichotomize thick and thin IMT to be comparable to that observed in a previous study, which used the per-SD difference in IMT values to estimate the atherosclerotic risks (3,33). We used overexpression of the tagged protein rather than the endogenous BRAP/NF-kB proteins in the Co-IP experiments. We acknowledge that overexpression might change protein cellular localization and thus the results might not truly reflect the protein–protein interaction in vivo. Further studies are warranted to validate our findings.

In summary, the results of the present study demonstrate that an SNP in BRAP confers a risk for carotid atherosclerosis. Through binding to the IKK signaling-some, BRAP enhances NF-kB nuclear translocation, initiates the transcription of downstream inflammatory cytokines and provokes the atherosclerotic process. Our results indicate that BRAP has an effect on cardiovascular disease. Further studies are warranted to identify treatment strategies for individuals at risk.

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DISCLOSURE
The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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