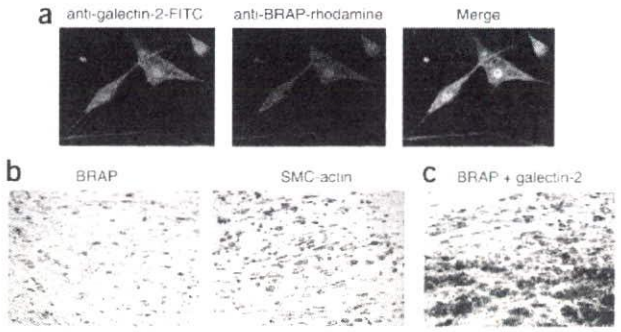

Figure 3 Colocalization of galectin-2 and BRAP in HCASMC and atherosclerotic lesion of coronary artery. (**a**) Colocalization of endogenous galectin-2 with BRAP in HCASMC. (**b**) Expression and colocalization of galectin-2 and BRAP in the coronary atherectomy specimen. Single-labeled immunohistochemistry of serial sections of primary atherosclerotic lesions from human coronary arteries obtained by directional coronary atherectomy, stained with anti-human BRAP or anti-human SMC-actin. Magnification, ×65.5 (BRAP) and ×90 (SMC-actin). (**c**) Double-labeled immunohistochemical staining with antibodies against BRAP (brown) and galectin-2 (blue). Magnification, × 89.5.

We examined the possibility of confounding effects by age, sex and classical risk factors within cases. We did not find any clear relationships between genotype and these factors (**Supplementary Tables 6** and 7 online), suggesting that the significant SNP in *BRAP* is an independent risk factor for myocardial infarction.

For assessment of stratification, we carried out genomic-control methods by genotyping 17 and 9 randomly selected SNPs for Japanese[13] and Taiwanese populations, respectively. The significance of stratification (P) and inflation factor ($\lambda_{3500,\ 4000}$ in Japanese and $\lambda_{350,\ 990}$ in Taiwanese) were 0.71 and 1.00 (95th percentile upper bound; 2.28) for Japanese, and 0.50 and 1.00 (95th percentile upper bound; 1.61) in Taiwanese, respectively. Because the maximum factor (χ^2 statistic for recessive model/χ^2 statistic for threshold) in this study was 10.44 in Japanese and 2.08 in Taiwanese, population stratification is unlikely as an explanation for our case-control association result in these populations.

Using antibodies directly labeled with fluorescein (anti-galectin-2) or rhodamine (anti-BRAP), we examined subcellular localization of native galectin-2 and BRAP in human coronary artery smooth muscle cells (HCASMC) and found that these proteins colocalized in the cytoplasm and nucleus (**Fig. 3a**). We also investigated whether the BRAP protein is in fact expressed in the myocardial infarction lesion, that is, the atherosclerotic lesion of the coronary artery. We detected immunoreactivities for BRAP in the SMCs and macrophages in atherosclerotic plaques (**Fig. 3b** and **Supplementary Fig. 1** online). Coexpression of BRAP and galectin-2 was also observed in most polymorphic SMCs and activated macrophages by double-labeled immunohistochemistry (**Fig. 3c**). We examined eight samples and all of them showed similar expression patterns.

The two SNPs in *BRAP* showing very strong associations did not cause amino acid substitutions. Therefore, we investigated whether these SNPs, rs11066001 (intron 3, 270A>G) and rs3782886 (exon 5, 90A>G; R241R), would affect *BRAP* expression by reporter gene analysis. A clone containing the intron 3 270A allele showed approximately half of the transcriptional activity of the 270G allele or that of the *BRAP* promoter only (**Fig. 4a**). No allelic difference was observed in constructs containing the exon 5 SNP (**Supplementary Fig. 2** online). To confirm these results, we cloned three tandem copies of the genomic segment including these SNPs, and obtained similar results (**Fig. 4a** and **Supplementary Fig. 2**). These results indicate that the substitution in intron 3, but not the one in exon 5, affects the transcription level of *BRAP*. We subsequently looked for nuclear factor(s) that might bind to oligonucleotides corresponding to genomic sequences of the 270A allele. No known protein was predicted to bind to this DNA segment by the TFSEARCH program (see URLs section in Methods) based on the TRANSFAC database[14]. Using nuclear extracts from HCASMC, we observed one band in the lane corresponding to the A allele, indicating binding of a nuclear protein(s) to the A allele (**Fig. 4b**). This result suggested that an unidentified nuclear factor(s) interacting with this genomic region might suppress transcription of *BRAP* and thereby have a role in the pathogenesis of myocardial infarction.

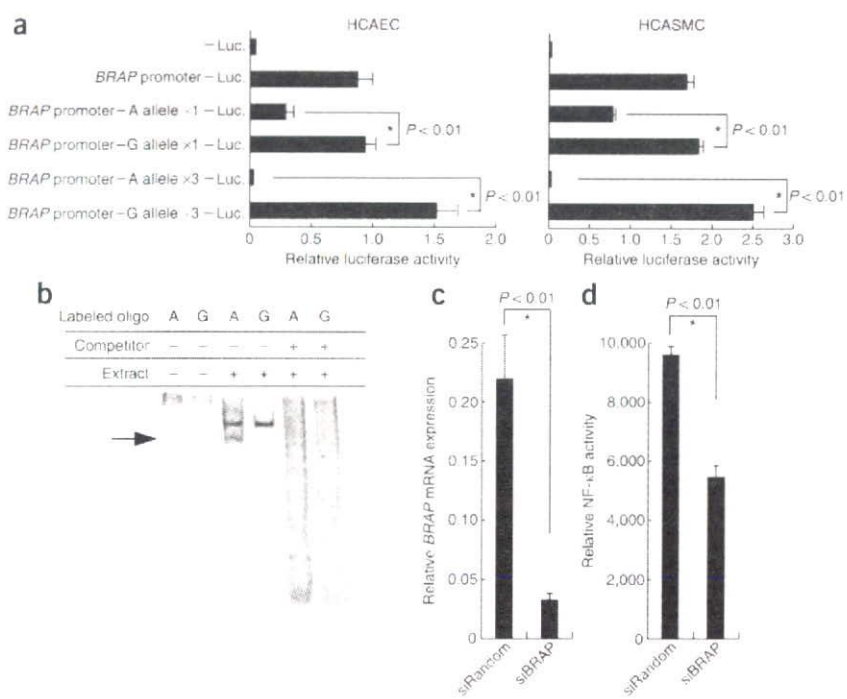

Figure 4 Functional analyses of the intron 3 SNP (rs11066001) of *BRAP*. (**a**) Transcriptional regulatory activity of intron 3 SNP of *BRAP* in HCASMC (left) and HCAEC (right). Each experiment was repeated three times and each sample was studied in triplicate. Luc, luciferase. (**b**) Binding of unknown nuclear factor(s) to intron 3 of *BRAP*. An arrow indicates the band showing specific binding of nuclear factor(s) to the A allele. (**c**) *BRAP* mRNA in random or siRNA-treated HCAEC. Each experiment was repeated three times and each sample was studied in duplicate. (**d**) Relative NF-κB activation in HCAEC. Each experiment was repeated three times and each sample was studied in triplicate. Asterisks (*) indicate data analyzed by Student's t-test. Error bars, s.d.

BRAP was originally identified as a protein that binds to the signal peptide of breast cancer suppressor protein BRCA1 (ref. 15), and is known to be an E3 ubiquitin ligase that associates with Ras and modulates mitogen activated protein (MAP) kinase signaling through regulation of the scaffolding activity of KSR (kinase suppressor of ras)[16]. The MAP kinase signaling pathway is well known as a regulator of cell survival, growth, differentiation, transformation and production of proinflammatory factors[17,18]. Galectin-2 has also been implicated in the inflammatory pathway through interactions with lymphotoxin-α and tubulin proteins[2]. Thus, we examined whether the cellular level of BRAP protein could influence NF-κB, a central transcription factor of inflammation[19]. siRNA against *BRAP* significantly suppressed *BRAP* mRNA (**Fig. 4c**), and resulted in inhibition of NF-κB activation in HCAEC (**Fig. 4d**). This result implied that altered expression of BRAP might influence the expression of genes involved in the NF-κB–dependent inflammatory pathway. Thus, it is conceivable that a higher expression of *BRAP* with an minor allele of intron 3 (G allele) might enhance the degree of inflammation through activation of NF-κB protein and thereby have an important role in the pathogenesis of myocardial infarction, although the relationship of BRAP protein with activation of coronary artery endothelial cells involved in plaque instability or rupture remains to be tested.

Coronary artery disease attributable to atherosclerosis is a leading cause of mortality in many countries[20]. We believe that our findings will motivate functional investigation of galectin-2 and BRAP, and also be useful for the development of novel diagnostic methods, treatments and prevention for this common but serious disorder.

METHODS

DNA samples. The diagnosis of definite myocardial infarction and clinical characteristics of three cohorts are described in **Supplementary Note** online. In brief, the Osaka Acute Coronary Insufficiency Study (OACIS) group recruited myocardial infarction cases in Japan[21]. For the Taiwanese population, subjects were recruited from the Kaohsiung Medical Hospital, Taiwan. All Taiwanese subjects are of Chinese descent. All study subjects provided written informed consent to participate in the study, or parental consent was obtained for subjects under 20 y old, according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama and by the Internal Review Board of the Kaohsiung Medical University Hospital, Kaohsiung.

SNP discovery and genotyping. Design of PCR primers, PCR experiments, DNA extraction, DNA sequencing, SNP discovery and genotyping of SNPs were described previously[1]. For SNP discovery, we searched the entire genomic region of *BRAP* (nt –5486 of 5′-flanking to nt 21 of 3′-flanking), except for those corresponding to repetitive sequences, by direct sequencing using ABI3700 sequencer (Applied Biosystems). For genotyping, we used the Invader assay[1] (rs11066001, rs3782886, rs3742001, intron 7 212 and rs10774633) and a direct sequencing method (rs601663 and rs12427276). For quality control of the Invader assay, the results were compared with those of direct sequencing; we examined 190 samples for each SNP and did not find any inconsistency between the results of Invader assay and direct sequencing.

Genetic association analyses. We selected tag SNPs using Haploview software[8] with a pairwise tagging mode and applied a permutation test for haplotype analysis. We also applied haplotype analysis using the program THESIAS[9] and conditional log-likelihood with Akaike information criterion (AIC): AIC = –2 × (the maximized value of the conditional log-likelihood) + 2 × (the number of parameters). As the number of parameters, we used the number of alleles or haplotypes with frequencies >0.01 that were used for each model. In the logistic regression analysis of a SNP, we first applied a 1-degree-of-freedom (1-d.f.) likelihood ratio test to determine whether a 1-d.f. multiplicative allelic effects model or a 2-d.f. full genotype model was more appropriate[9]. Because we did not find any significant difference from the full genotype model ($P > 0.05$), we assumed a multiplicative allelic effects mode. Next, we carried out a forward logistic regression analysis, where we started by assessing whether the most significant SNP was sufficient to model the association among the SNP set. For this, we used a 1-d.f. likelihood ratio test for adding each of the remaining SNPs to the model by assuming multiplicative allelic effects for the additional SNPs.

Because subjects for the stepwise sequencing and genotyping were randomly selected from the Japanese samples, we strictly applied Bonferroni's correction for the number of SNPs that were identified from the SNP discovery phase. When we analyzed the combinatorial effect of rs11066001 (*BRAP*), rs1041981 (*LTA*) and rs7291467 (*LGALS2*) on myocardial infarction susceptibility, we first applied a 1-d.f. likelihood ratio test to determine whether a 1-d.f. multiplicative allelic effects model or a 2-d.f. full genotype model was more appropriate[12]. Because we found no significant difference from the full genotype model ($P > 0.05$), we considered a multiplicative allelic effects model to be more appropriate. Then we applied a 1-d.f. likelihood ratio test for adding the remaining SNP to each of the one-SNP models by assuming multiplicative allelic effects for the additional SNPs to assess whether the SNPs were themselves associated with myocardial infarction. Furthermore, we carried out a 1-d.f. likelihood ratio test for adding a statistical interaction term between the SNPs.

Relationships between clinical profiles and genotype information of the cases was examined by χ^2 test for sex difference, one-sided Fisher's probability test for coronary risk factors, and one-way ANOVA for quantitative clinical parameters.

Tandem affinity purification. The tandem affinity purification procedure was carried out essentially as previously described[7], with some modifications[2].

Coimmunoprecipitation experiment. For coimmunoprecipitation in COS7 cells, we transfected an expression plasmid of Myc- or S-tagged BRAP or galectin-2 into COS7 cells (HSRRB; JCRB9127) using Fugene6 (Roche). Immunoprecipitations were done in lysis buffer (20 mM Tris pH 7.5, with 150 mM NaCl, 0.4% Nonidet P-40 containing 5 μg/ml of MG-132 and protease inhibitor tablet EDTA- Roche). At 24 h after transfection, cells were lysed, and immunoprecipitations were done using anti-Myc tag (Santa Cruz) or S-protein agarose (Novagen). We visualized the immune complex using HRP conjugated S-protein (Novagen) or anti-Myc antibody peroxidase conjugate (Santa Cruz).

Luciferase assay. To investigate functions of two SNPs (intron 3 270A>G and exon 5 50A>G) in *BRAP*, we first cloned genomic fragments for the *BRAP* promoter region (nt –992 of 5′-flanking to 21 of exon 1) into pGL3-basic vector (Promega) in the 5′-to-3′ orientation (*BRAP* promoter-luciferase). Then, double-stranded oligonucleotides (nt 262–279 of intron 3 and nt 83–100 of exon 5) were cloned into the *BRAP* promoter–luciferase vector. We also constructed three tandem copies of the oligonucleotides for each SNP region into the *BRAP* promoter–luciferase vector. We transfected these constructs in human coronary artery smooth muscle cells (HCASMC) and human coronary artery endothelial cells (HCAEC) (CAMBREX) using nucleofector system (Amaxa). We then carried out the luciferase assay according to the manufacturer's protocol. We lysed the cells in passive lysis buffer (Promega) 24 h after transfection and then measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Each experiment was independently repeated three times and each sample was studied in triplicate. Statistical analyses were done using Student's *t*-test.

Electrophoretic mobility shift assay. We prepared nuclear extracts from HCSMC as previously described[22] and then incubated in the presence of 1% bovine serum albumin and absence of poly d(I-C) with six tandem copies of oligonucleotides, corresponding to nt 254–278 of intron 3, labeled with digoxigenen -11-ddUTP using the digoxigenin gel-shift kit (Roche). For competition studies, we preincubated nuclear extract with unlabeled oligonucleotide (100-fold excess) before adding digoxigenin-labeled oligonucleotide. We separated the protein–DNA complexes on a nondenaturing 7% polyacrylamide gel in 0.5× Tris-Borate-EDTA buffer. We transferred the gel to nitrocellulose membrane and detected the signal with a chemiluminescent detection system (Roche) according to the manufacturer's instructions.

Confocal microscopy. Polyclonal antisera against human BRAP were raised in rabbits using recombinant partial BRAP protein (amino acids 1–134) synthesized in *Escherichia coli* using the pET29 system (Novagen). The antisera showed no cross-reactivity to the other proteins by protein blot analyses using protein extracts of HCASMC. Polyclonal antisera against BRAP and antisera against human galectin-2 (ref. 2) were directly labeled with fluorescein and rhodamine, respectively, using EZ label fluorescein or rhodamine protein labeling kit (PIERCE). HCASMCs were fixed, and they were subsequently incubated with the antibodies in phosphate-buffered saline containing 3% bovine serum albumin. We observed specimens using OLYMPUS FLUOVIEW confocal laser scanning microscope (OLYMPUS).

RNAi experiments. Double-strand stealth RNAi oligonucleotides (BRAP-HS112138 for *BRAP* and 12935-400 for negative control) were purchased from Invitrogen. The oligonucleotides were transfected in HCAEC using the nucleofector system (Amaxa), and 24 h after transfection each RNAi was re-transfected with pNiFty plasmid vector, a luciferase reporter vector containing NF-κB–specific E-selectin promoter (Invivogen), by nucleofector system (Amaxa). After 24 h, the cells were collected and luciferase activity was measured using the Dual-Luciferase Reporter Assay System. We isolated total RNA using RNAiso (Takara). cDNA was prepared from 2 μg of total RNA by dT_{15} priming and synthesized using SuperScript reverse transcriptase (Life Technologies). We quantified mRNA using the LightCycler 480 SYBR Green PCR kit (Roche) and a machine LightCycler 480 (Roche). Each experiment was independently repeated three times and each sample was studied in triplicate. Statistical analyses were done using Student's *t*-test.

Immunohistochemistry. We collected tissue samples from individuals with myocardial infarction by elective directional coronary atherectomy after obtaining informed consent. Immunohistochemical protocols were carried out as described previously using rabbit polyclonal antibodies to human BRAP and galectin-2 (ref. 2).

URLs. TFSEARCH program, http://www.cbrc.jp/research/db/TFSEARCHJ.html.

Accession codes. GenBank: BRCA1-associated protein (*BRAP*), NM_006768.2.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

K. Ozaki performed most of the experiments and wrote the manuscript; H.S., Y.S., H.M., T.-H.L., Y.M., S.-H.S., S.I., S.-H.H.J. and M.H. managed DNA samples and clinical information; K.I., K. Odashiro and M.N. performed the immunohistochemistry; T. Tsunoda performed the statistical analyses; A.A. and Y.O. performed the data analyses; Y.N. contributed to SNP discovery and preparation of the manuscript; and T. Tanaka supervised this study.

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