A functional SNP in **EDG2** increases susceptibility to knee osteoarthritis in Japanese

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Osteoarthritis (OA) is the most common form of arthritis and is characterized by the gradual loss of articular cartilage. Several OA-susceptibility genes have been identified; however, there are few pharmaceutical targets that can be targeted with small-molecule compounds. To investigate whether a susceptibility gene for OA exists among G-protein-coupled receptors (GPCRs), we performed a stepwise association study for 167 single nucleotide polymorphisms (SNPs) in 44 GPCR genes that were present in cartilage. Through the stepwise association study, an SNP located in the promoter region of **EDG2** [endothelial differentiation, lysophosphatidic acid (LPA) GPCR, 2] (2,820G/A; rs10980705) showed significant association with knee OA in two independent populations (pooled $P = 2.6 \times 10^{-5}$). Luciferase and electrophoretic mobility shift assays indicate that this SNP exerts an allelic difference on transcriptional activity and DNA binding in synovial cells, with the susceptibility allele showing increased activity and binding. **EDG2** encodes an LPA receptor dominantly expressed in the synovium. The LPA receptor increased the expression of inflammatory cytokines and matrix metalloproteases in synovial cells. Our findings suggest that the LPA–EDG2 signal is involved in the pathogenesis of OA via catabolic process.

INTRODUCTION

Osteoarthritis (OA, MIM 165720) is the most common form of arthritis and is characterized by the gradual loss of articular cartilage. It causes pain and dysfunction of joints, and is becoming a major burden on the aging society (1,2). Millions of people are suffering from this disease; however, there is no good medical treatment for it. We have only a few pharmaceutical options such as oral analgesics, topical steroids and hyaluronic (3). Development of innovative drugs has long been awaited. Searching for drug target molecules in the genome is a promising approach.

Epidemiological and genetic studies have shown that OA has a genetic component (4,5). Identification of disease-susceptibility genes brings us not only a better understanding of the pathogenesis of OA, but also new therapeutic targets.

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Several OA-susceptibility genes have been identified (6); however, there are few pharmaceutical targets that can be targeted with small-molecule compounds. Currently, a huge gap exists between the identification of susceptibility genes and the finding of ‘druggable’ targets.

The seven transmembrane G-protein-coupled receptor (GPCR) families have been considered a good druggable target because >30% of marketed drugs interact with GPCRs (7). To move on to the final goal of drug invention efficiently and swiftly, we planned to search susceptibility genes for OA among GPCRs. In this study, we found that an SNP located on the promoter region of EDG2 [endothelial differentiation, lyso-phosphatidic acid (LPA) GPCR, 2] was associated with knee OA by stepwise association study. Functional analysis revealed that the OA susceptibility allele increased its promoter activity. Furthermore, we found LPA–EDG2 signal induced inflammatory cytokine and matrix metalloproteases (MMPs) gene in synovial cells. Therefore, our findings bring a novel therapeutic target for OA treatment.

RESULTS

Identification of landmark SNP that associate with knee OA

We first selected 64 GPCR candidates expressed in the cartilage as determined by micro-array analysis (data not shown). Among these, we genotyped 167 SNPs for 44 genes selected as described in Materials and Methods (Supplementary Material, Table S1). We then examined the association between these SNPs and knee OA using 368 individuals with OA among GPCRs. In this study, we found that an SNP located on the promoter region of EDG2 had allele frequencies for these 155 SNPs (the first screen) showed a significant association (P < 0.05) for 13 SNPs. To confirm the association, we genotyped these 13 SNPs in an independent case–control population comprising 276 individuals with knee OA and 323 controls. Among 167 SNPs, 160 SNPs were polymorphic, and 155 of these were in Hardy–Weinberg equilibrium. Comparison of genotypic and allelic frequencies for these 155 SNPs showed a significant association (P < 0.05) for 13 SNPs. To confirm the association, we genotyped these 13 SNPs in an independent case–control population comprising 276 individuals with knee OA and 323 controls. Finally, we found only one SNP (rs3739708) showed a significant association in a recessive model in two independent populations (Supplementary Material, Table S2).

Linkage disequilibrium mapping and identification of OA susceptibility SNP

To localize the susceptibility gene, we evaluated the linkage disequilibrium (LD) extension with the landmark SNP. We genotyped 368 individuals with knee OA for SNPs in the 800 kb region around rs3739708 and examined the LD index (D') between rs3739708 and SNPs that had allele frequencies >10%. D' scores were decreased at rs11794726 (D' = 0.69) and rs12353088 (D' = 0.40), which are located 35 kb upstream and 10 kb downstream of rs3739708, respectively (Supplementary Material, Fig. S1A), limiting the LD extension with rs3739708 to a 45 kb region.

To identify a disease-causing variation, we first re-sequenced this 45 kb region, except for repetitive genomic sequences, in 48 individuals with knee OA. We identified a total of 27 polymorphisms (Supplementary Material, Fig. S1B, Table S3). We genotyped these 27 polymorphisms in individuals with knee OA used in the first screen, and constructed a pairwise LD structure using polymorphisms having allele frequencies >10% (Supplementary Material, Fig. S1C). These polymorphisms had high D' scores (>0.9), indicating that disease-causing variations could exist among these 27 polymorphisms. We next examined the association of all of 27 polymorphisms using 644 knee OA patients and 640 controls. We identified three SNPs that had an association more significant than rs3739708 (i-EDG2-25) in the recessive model (Table 1). Moreover, we examined haplotypes based on the 13 SNPs having allele frequencies >10% within the 45 kb region. Eight haplotypes with frequencies >1% represented >95% of both case and control populations. The haplotype association was much less significant than that of the landmark SNP alone (Supplementary Material, Table S4). Thus, the presence of a hidden ungenotyped sequence variation is unlikely. We then tested the association of the three SNPs and i-EDG2-25 in resident-cohort populations. The association of EDG2 with knee OA was reproduced in the independent populations: three SNPs, i-EDG2-9 and -12 located on the 5'-flanking sequence and i-EDG2-25 located on the intron 1 of EDG2, showed significant association (Table 2). Because i-EDG2-9 showed higher association and odds ratio than i-EDG2-12 and 25, we considered i-EDG2-9 to be the disease-causing SNP. The homozygotic A allele was over-represented in the knee OA population. The final P-value for the association of the SNP was calculated as 2.6 × 10^{-5} (odds ratio = 2.3; 95% CI = 1.6–3.3) by using a Mantel–Haenszel analysis on two independent populations (case–control and resident-cohort populations), which was still significant after conservative Bonferroni’s correction (using the number of SNPs in first screen, 155 tests).

We checked the effects of confounding factors such as age and body mass index (BMI) to evaluate whether they could make a pseudo-positive association. There was no significant difference in mean age or BMI for the SNP i-EDG2-9 (Supplementary Material, Table S5).

To gain insight into the role of EDG2 in OA, we analyzed its expression in cartilage and synovium from knee OA patients by quantitative real-time PCR (Supplementary Material, Fig. S2). EDG2 expression was significantly higher in the synovium than in the cartilage. EDG2 is an LPA receptor. There are five identified LPA receptors in mammals (EDG2, EDG4, EDG7, GPR23 and GPR92). LPA receptors are involved in various cellular functions such as cell survival, proliferation and migration through binding its intrinsic ligand, LPA (8). EDG2 expression in the synovium was higher than that of other LPA receptors (Supplementary Material, Fig. S2). These results suggest that EDG2 is a critical LPA receptor in the synovium.

Functional analysis of susceptibility SNP

i-EDG2-9 is located in a putative promoter region (2,820 bp upstream of the transcription start site) and is located immediately after a putative AP-1 binding motif (TGGAGCTA). Therefore, we hypothesized that i-EDG2-9 may alter EDG2 transcriptional activity. We transiently transfected a synovial cell line E11 (9) with vectors containing five tandem copies of the sequences surrounding i-EDG2-9 coupled to a luciferase
reporter gene. As an inducer of AP-1, we used phorbol myristate acetate (PMA), which is known to increase AP-1-binding activity in synovial cells (10). PMA stimulation increased luciferase activity, which was higher in cells transfected with the vector harboring the susceptibility A allele (Fig. 1A).

We then examined the allelic difference in the binding of i-EDG2-9 to trans factors by electrophoretic mobility shift assay (EMSA). PMA stimulation increased AP-1 transcriptional activity in E11 cells in a dose-dependent manner (Supplementary Material, Fig. S3). Nuclear extracts from E11 cells stimulated by PMA formed DNA–protein complexes with oligonucleotides harboring i-EDG2-9 in a time-dependent manner (Fig. 1B). The band intensity for DNA–protein complexes derived from the A allele was higher than that from the G allele (Fig. 1C). The complex was diminished by excess amounts of a non-labeled oligonucleotide with the AP-1 consensus sequence. These data suggest that the EDG2 promoter harboring the A allele tends to express higher levels of EDG2 transcripts owing to a stronger binding affinity for AP-1.

The role of LPA/EDG2 system in synovium

To examine the role of LPA/EDG2 signaling in synovium, we stimulated primary fibroblast-like cells derived from the inflamed synovial tissue of an OA patient (HFLS-OA) by LPA and examined the gene expression of inflammatory cytokines and MMPs involved in the catabolic process of OA (11). LPA stimulation resulted in an early and transient expression increase of the inflammatory cytokine genes for interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6 (Fig. 2A–C), as well as upregulation of MMP genes (Fig. 2D–F). LPA treatment induced the inflammatory cytokine genes in a dose-dependent manner (Fig. 2G–I). The induction was inhibited by treatment with a pharmacological EDG2 antagonist, Ki16425 (Fig. 2J), and by introducing siRNA targeting EDG2 into HFLS-OA (Fig. 2K, L). These findings indicate that EDG2 is critical for the catabolic response in synovial tissue caused by LPA signaling.

DISCUSSION

We have shown that a functional SNP in the EDG2 region is associated with knee OA. We have further demonstrated that this SNP affects AP-1-mediated transcriptional activity, which may result in increased EDG2 expression when the allele is over-represented in knee OA patients. Expressional and functional analyses revealed that EDG2 is the critical LPA receptor in synovium. OA is not just a cartilage disease, but also affects the entire joint structure including the synovial membrane, subchondral bone and ligament (12). To our knowledge, however, EDG2 is the first OA-related gene to be primarily expressed in the synovium, highlighting the importance of the synovium in the pathogenesis of OA.

In the OA synovium, inflammatory changes occur including synovial hypertrophy and hyperplasia with an increased

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a11, 12 and 22 indicate homozygotic for the minor allele, and heterozygotic and homozygotic for the major allele, respectively.
bLandmark SNP.
number of lining cells (13). Also, the production of inflammatory cytokines such as IL-1β, TNF-α and cartilage-degrading enzymes such as the MMPs is enhanced (14, 15). Inflammatory cytokines and MMPs released from the synovium affect the articular cartilage in a paracrine manner via the synovial fluid, and they promote a catabolic process that leads to progressive cartilage degradation. We have shown here that LPA induces both inflammatory cytokines and MMPs in synovial fibroblasts via EDG2, suggesting that LPA/EDG2 signaling is involved in the pathogenesis of OA via this catabolic process. Our findings could therefore provide new therapeutic options for OA using druggable targets.

MATERIALS AND METHODS

Selection of SNPs

We selected 64 GPCRs expressed in the cartilage based on GeneChip (Affymetrix) analysis using total cartilage RNAs from eight OA and eight normal individuals described previously (16). We excluded one gene located on the X chromosome from further study. Next, we estimated haplotype-tagging of SNPs for the 63 cartilage GPCR genes located on autosomal chromosomes using large-scale genotype data described elsewhere (17). We excluded genes not having any Invader probe labeling or showing a minor allele frequency of no >10%. We determined the haplotype block structure of each gene by a method reported by Gabriel et al. (18), and selected SNPs that represented any haplotype having a frequency >0.1% in each haplotype block. Finally, we selected 167 SNPs for 44 genes (Supplementary Material, Table S1).

SNP discovery and genotyping

For a fine-scale association study and LD mapping of the 45 kb region containing exon 1 of EDG2, we detected SNPs by direct sequencing of genomic DNA from 48 affected individuals as described previously (19, 20). We genotyped the SNPs using the Invader assay, TaqMan assay, and direct sequencing of PCR products with capillary sequencers (ABI3700, Applied biosystems), and then tested for association.

Table 2. Association test in resident-cohort population

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a11, 12 and 22 indicate homozygotic for the minor allele, and heterozygotic and homozygotic for the major allele, respectively.
bP-value and odds ratio were calculated using the recessive model.
cEDG2-25 is identical to rs3739708, the landmark SNP.

Subjects

All individuals recruited for this study were Japanese and received clinical and radiographic examination by orthopedic specialists. The selection method and criteria were described previously (16). We recruited case–control subjects from individuals who lived in mainland Japan (Honshu), and visited the participating clinical institutions. We studied 644 individuals with knee OA (83% female; mean age ± SD = 71.7 ± 7.7 years) and 640 controls (36% female; mean age ± SD = 61.5 ± 10.3 years). In the second screen of the stepwise screening, we used genotype data of another 654 controls from a previous study (45% female; mean age ± SD = 48.5 ± 21.6 years) (17). The minor allele’s homozygote frequencies for the landmark SNP (rs3739708) in these two controls were almost the same (3.4 and 3.5%, respectively). All affected individuals were symptomatic and were treated in the participating institutions on a regular basis.

We also recruited resident-cohorts from inhabitants of Miyagawa village and Nansei town in Mie prefecture, which is located in the middle of mainland Japan. For each individual, we took standard three-direction knee radiographs. Using the KL grade and self-reported pain, we classified Miyagawa village subjects into OA (KL grade 2–4) and non-OA (KL grade 0–1 and without self-reported pain) groups. We also classified Nansei town subjects into OA (JSN grade 2–5) and non-OA (KL grade 1 and without self-reported pain) groups. The OA group consisted of 238 subjects (75% female; mean age ± SD = 71.5 ± 7.8 years) and the non-OA group consisted of 279 subjects (62% female; mean age ± SD = 68.8 ± 8.1 years). Other clinical parameters were described previously (16).

The study protocol was approved by the ethical committees of the SNP Research Center of RIKEN and the participating institutions, and written informed consent was obtained from each participant. We obtained blood samples from the participants and prepared genomic DNA from peripheral leukocytes in accordance with standard protocols.

Association analysis

We used case–control subjects for a stepwise association study and genotyped 167 SNPs in genomic DNA from 368 individuals with knee OA and 323 control individuals. For the successfully genotyped SNPs, which were in Hardy–Weinberg equilibrium in the control population, we calculated
P-values as previously described (the first screen) (21). SNPs that passed the first screen were further genotyped in a second replication panel consisting of 276 individuals with knee OA and the association was tested using control genotype data of 654 individuals that were genotyped in a previously study (17) as an independent test (the second screen). For both screens, P-values < 0.05 were considered significant.

After identification of the landmark SNP through the stepwise screening followed by LD mapping and re-sequence of the LD region containing the SNP (rs3739708), all discovered SNPs within the region were genotyped for a total of 644 OA cases (all the cases of the first and second screens) and 640 controls (323 controls of the first screen and 317 controls further recruited). Then, we calculated the final P-value of the case–control population using the genotype data.

**Human articular cartilage and synovium samples**

We obtained cartilage and synovium from individuals with knee OA during surgery (total knee arthroplasty). These samples were immediately frozen in liquid nitrogen and stored at −80°C.

**Statistical analysis**

We carried out statistical analysis for the association study, Hardy–Weinberg equilibrium, calculation of LD coefficients (D') and LD index (Δ) as described previously (21,22). We estimated haplotype frequencies using the expectation–maximization algorithm (23). We performed a Mantel–Haenszel analysis to calculate the pooled P-value and odds ratio of two independent association studies (case–control and resident-cohort). Luciferase assay data were analyzed by Student’s t-test using Excel software (Microsoft).

**Cell culture**

HFLS-OA, primary fibroblast-like cells derived from the inflamed synovial tissue of an OA patient, was purchased from Cell Applications. HFLS-OA was routinely cultured in synoviocyte growth medium (Cell Applications). We used these cells within five passages. For the gene expression assay, cells were plated on a 24-well plate at 1 × 10^5 cells/well in synoviocyte growth medium. After reaching confluence, cells were cultured in Ham’s F-12 medium (Invitrogen) containing 0.1% fatty acid-free BSA (Bovine serum albumin, Sigma) for 12 h, and then stimulated by LPA (1-oleyl-sn-glycerol-3-phosphate, Sigma) with or without an LPA1/3 antagonist Ki16425 (Sigma).

For the luciferase assay and EMSA, we used E11, a fibroblast-like synovial cell line (9). We grew E11 cells in Dulbecco’s Modified Eagle’s medium (Sigma) supplemented with 10% FBS (Fetal bovine serum) containing 2 mM L-glutamine and antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin).

**siRNA**

The siRNA directed against human EDG2 was designed using the siRNA design support system in the Takara Bio website (http://www.takara-bio.co.jp). As a control, we used a scrambled siRNA targeted against asporin as described elsewhere (24). The siRNA and control were synthesized by Takara Bio. Oligonucleotide sequences are listed in Supplementary Material, Table S6. We transfected siRNAs into HFLS-OA cells using TransIT TKO (Mirus), and cultured the cells for 36 h in complete synoviocyte growth medium. We cultured the cells in Ham’s F-12 medium containing 0.1% fatty acid-free BSA for a further 12 h, and then the stimulated cells by application of 10 μM LPA for 2 h.
Real-time PCR

We extracted total RNA from clinical samples or cells using Isogen (Nippongene) and purified them using an SV-Total RNA Isolation system (Promega). We synthesized first-strand cDNA. We carried out real-time PCR on an ABI PRISM 7700 sequence detection system (Applied biosystems) using QuantiTect SYBR Green PCR (Qiagen) according to the manufacturer’s instructions. First-strand cDNA was amplified using primers specific for LPA receptor genes (EDG2, EDG4, EDG7 and GPR23) or pro-inflammatory cytokine genes (TNFA, IL1B and IL6) and G3PDH (Toyobo). The expression

Figure 2. Induction of pro-inflammatory cytokines and MMPs by LPA. Real-time PCR in a human synovial cell line, HFLS-OA. (A–F) Time course of induction by 10 mM LPA for pro-inflammatory cytokine genes (A) TNFA, (B) IL1B and (C) IL6 and MMP genes (D) MMP1, (E) MMP3 and (F) MMP13. (G–I) Dose-dependent induction by LPA of pro-inflammatory cytokine genes (G) TNFA, (H) IL1B and (I) IL6. (J–L) Inhibition of LPA-induced IL6 expression by an antagonist, Ki16425 (J) and siRNA (K) targeting EDG2 in HFLS-OA cells. Decreased expression of EDG2 was confirmed (L). Data show the mean ± SEM of triplicate or quadruplicate assay.
of LPA receptor genes and pro-inflammatory cytokine genes was normalized to that of G3PDH from the same cDNA using a standard curve method described by the manufacturer. Specific primer sequences are listed in Supplementary Material, Table S7.

Electrophoretic mobility shift assay
We grew E11 cells until confluency and then cultured them with 10 ng/ml PMA (Sigma) for 0–8 h. We prepared a nuclear extract from these cells as previously described (25). We incubated the nuclear extract with 36 bp double-strand oligonucleotide probes for i-EDG2-9 alleles (G and A) for 20 min at room temperature. Oligonucleotide sequences are listed in Supplementary Material, Table S6. The AP-1 oligonucleotide sequence was described elsewhere (26). Probes were labeled using the digoxigenin gel-shift kit (Roche). For competition studies, we pre-incubated the nuclear extract with unlabeled oligonucleotides (125-fold excess) before adding the labeled oligonucleotide. Protein–DNA complexes were separated by electrophoresis on a 6% polyacrylamide gel in 0.5× TBE (Tris-borate-EDTA) buffer, followed by transfer to a nitrocellulose membrane and detection using a chemiluminescent signal detection system (Roche).

Luciferase assay
We constructed a luciferase reporter plasmid by cloning five connected copies of the adjacent 36 bp double-strand oligonucleotides containing the i-EDG2-9 allele into the pGL3-promoter vector (Promega) upstream of the SV40 promoter as described previously (27). We transfected E11 cells (5 × 10⁴ cells/well) with 0.2 µg of the constructs and 4 ng of pRL-TK vector (Promega) as an internal control for transfection efficiency using Fugene-6 (Roche). After 24 h, we cultured the transfected cells with or without 10 ng/ml of PMA. After 5 h, cells were solubilized and luciferase activity was measured using the Pikkagen dual luciferase assay system (Toyo Ink, Tokyo, Japan).

Accession numbers
Human EDG2 mRNA, NM_057159.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

REFERENCES


