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Genome-wide Association Scan Identifies a Prostaglandin-Endoperoxide Synthase 2 Variant Involved in Risk of Knee Osteoarthritis

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Osteoarthritis (OA), the most prevalent form of arthritis in the elderly, is characterized by the degradation of articular cartilage and has a strong genetic component. Our aim was to identify genetic variants involved in risk of knee OA in women. A pooled genome-wide association scan with the Illumina550 Duo array was performed in 255 controls and 387 cases. Twenty-eight variants with $p < 1 \times 10^{-5}$ were estimated to have probabilities of being false positives ≤ 0.5 and were genotyped individually in the original samples and in replication cohorts from the UK and the U.S. (599 and 272 cases, 1530 and 258 controls, respectively). The top seven associations were subsequently tested in samples from the Netherlands (306 cases and 584 controls). rs4140564 on chromosome 1 mapping 5′ to both the *PTGS2* and *PLA2G4A* genes was associated with risk of knee OA in all the cohorts studied (overall odds ratio $OR_{MH} = 1.55$ 95% C.I. 1.30–1.85, $p < 6.9 \times 10^{-7}$). Differential allelic expression analysis of *PTGS2* with mRNA extracted from the cartilage of joint-replacement surgery OA patients revealed a significant difference in allelic expression ($p < 1.0 \times 10^{-6}$). These results suggest the existence of *cis*-acting regulatory polymorphisms that are in, or near to, *PTGS2* and in modest linkage disequilibrium with rs4140564. Our results and previous studies on the role of the cyclooxygenase 2 enzyme encoded by *PTGS2* underscore the importance of this signaling pathway in the pathogenesis of knee OA.

Introduction

Osteoarthritis (OA [MIM 165720]) of the knee is a common complex disorder resulting in joint disability with known constitutional and environmental risk factors for development and progression, such as age, obesity, hormonal status, bone density, physical activity, and past history of trauma.[1] Knee OA also has an important genetic component, and several studies have investigated the role of candidate genes in the risk of hip and knee OA. Several genes with common polymorphisms consistently affecting risk of OA have been reported to date (e.g., [2–6]). In general, the genetic variants involved do not have large attributable risks. Rather, in common with other complex traits, the increased risks for carrying a predisposing genetic variant appear to be fairly modest, with most of them having odds ratios between 1.3 and 2.0, suggesting that a large number of genes each with relatively modest effect are contributing to the genetic etiology of OA.[7]

To date, two large case-control association scans have been reported. Mototani and coworkers[8] tested 72,000 markers for association with hip OA, and identified a variant in the calmodulin 1 (*CALM1* [MIM 114180]) gene to be strongly associated in the Japanese population. However,

studies in UK samples failed to show an association of this variant with hip[9] or knee OA.[3] Spector and coworkers[10] examined 25,000 genic SNPs for association with radiographic knee osteoarthritis in men and women from the UK and identified a SNP in a gene of unknown function (the leucine-rich repeats and calponin homology domain-containing 1, *LRCH1* [MIM 610368]). That genetic variant has failed to show an association in subsequent studies in both Asian and European samples.[11,12] To our knowledge, to date no large-scale genome-wide association scan with extensive coverage (i.e., 100,000 or more markers) of knee OA has been carried out.

In this study, we report a large-coverage pooled genome-wide association scan (GWAS) of knee OA and the results of successively testing individually the most highly associated SNPs in five case-control studies.

Material and Methods

Study Subjects

We combined data from five independent cohorts into a discovery sample, a UK replication cohort, a U.S. replication cohort, and a Dutch replication cohort (see Figure 1). The numbers of cases and controls from each cohort used in the discovery and

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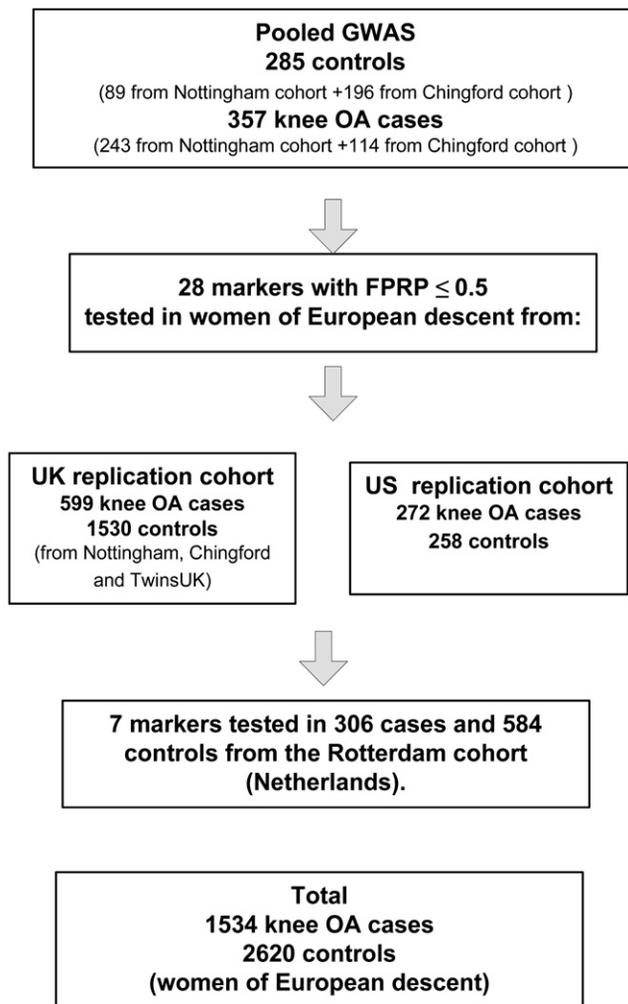*Correspondence: ana.valdes@kcl.ac.uk

Figure 1. Study Strategy Used for Discovery and Replication

replication samples is shown in Table 1. All study subjects were of self-reported white ethnicity.

Definitions of OA

All affected individuals taking part in this study had standardized extended weight bearing anteroposterior radiographs of their knees. Two different definitions of OA were used, clinical OA for the UK and US case controls and radiographic OA for the TwinsUK, Chingford, and Rotterdam population-based studies (Table 1).

Clinical OA was defined by American College of Rheumatology criteria,[13] namely, patients had knee pain due to OA on most days during at least 1 month in a 3 month period prior to screening, with the addition of at least one of the following: age >50 years, morning knee stiffness lasting >30 min, or knee crepitus.

For radiographic OA, the description of scoring of the radiographs is described elsewhere in detail.[14,15] Radiographs were scored for the presence of radiographic OA (ROA) of the knee according to the Kellgren/Lawrence (K/L) score.[16] Knee OA was defined as a K/L score ≥2 of one or both joints.

The number of patients and controls from each cohort taking part in the discovery and replication studies, the diagnosis criteria, and their descriptive characteristics are shown in Table 1. Specific characteristics of each cohort are summarized below.

UK Case-Control Study
Women affected by knee OA cases were recruited in Nottingham both from families with a history of OA and from clinic populations. All research participants gave written informed consent to take part. The study protocol was approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee.

U.S. Case-Control Study
Female knee OA patients attending clinics in the mid-Atlantic region of the U.S. (Maryland and North Carolina) provided written informed consent before entering the study, which was conducted in accordance with International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guidelines for Good Clinical Practice and was administered by the institutional review boards. Controls were U.S. white women over the age of 50 not diagnosed for OA.

The Rotterdam Study
The Rotterdam study is a prospective, population-based cohort investigating determinants, incidence, and progression of chronic disabling diseases in the elderly. The rationale and study design have been described previously.[17] The medical ethics committee of Erasmus University Medical School approved the study, and written informed consent was obtained from each participant.

Table 1. Descriptive Characteristics of Study Subjects by Cohort of Origin

Cohort (Country of Origin)	Diagnosis Criteria	Number in Discovery Sample (Pooled GWAS)	Number in Replication Studies	Number in Meta-analysis	Age Years (SD)	BMI kg/m^2 (SD)
Chingford study (UK)	Population-based, radiographic knee OA	114 F^a OA, 196 F controls	146 F OA, 243 F controls	260 F OA, 439 F controls	64.0 years (SD = 6.0)	26.7 kg/m^2 (SD = 4.6).
Case control (UK)	Clinical knee OA and age-matched controls	243 F OA, 52 F controls, 37 M^a controls	258 F OA, 402 F controls	501 F OA, 454 F OA	73.0 years (SD = 8.5)	27.7 kg/m^2 (SD = 4.7)
TwinsUK (UK)	Population-based, radiographic knee OA	0	195 F OA, 885 F controls	165^b F OA, 679^b F controls	65.0 years (SD = 13.1)	25.1 kg/m^2 (SD = 4.4)
Case control (US)	Clinical knee OA and age-matched controls	0	272 F OA, 258 F controls	272 F OA, 258 F controls	60.5 years (SD = 8.9)	28.8 kg/m^2 (SD = 4.9)
Rotterdam study (Netherlands)	Population-based, radiographic knee OA	0	306 F OA, 584 F controls	306 F OA, 584 F controls	68.3 years (SD = 8.0)	26.7 kg/m^2 (SD = 4.1)

^{a} F, female; M, male.
^{b} Number of singletons.

The Chingford Study

The Chingford study is a prospective population-based longitudinal cohort of women who were derived from the age/sex register of a large general practice in North London and who are representative of the general UK population in terms of weight, height, and smoking characteristics.[18] The study design and rationale are described elsewhere in detail.[19] The Guys and St Thomas' Trust and Waltham Forest Trust ethics committees approved the study protocol. After study procedures were explained to participants, they gave written consent.

TwinsUK

Participants were derived from the TwinsUK Adult Twin Registry (Twin Research and Genetic Epidemiology Unit, St Thomas Hospital, London, UK), a volunteer sample previously developed to study the heritability and genetics of age-related diseases. These twins, without selecting for particular diseases or traits, were recruited from the general population through a series of national media campaigns in the UK[20] and were shown to be comparable with age-matched population singletons in terms of disease-related and lifestyle characteristics.[21] The study was approved by St Thomas' Hospital Research Ethics Committee, and all twins provided informed written consent.

Statistical Methods

Detection of Association from Pools

The difference in allele frequency between pools was expressed as a Z score given by $z = |f_{cases} - f_{controls}|/\sigma$ where f_{cases} is the estimated frequency of a SNP allele in cases, $f_{controls}$ is the same in controls, and σ is a standard deviation given by both the sampling error and the error owing to imprecise measurement of the allele frequency in a pool as described in detail in [22].

False-Positive Report Probabilities

False-positive report probabilities were computed for the highest Z scores coming out of the GWAS pooled analysis as described by Wacholder et al.[23] with the estimated allele frequencies from pools to estimate odds ratios and confidence intervals and the p value corresponding to the Z scores.

Individual Polymorphism Genetic Associations

The association between individual SNP genotypes and OA was tested by comparing SNP allele frequencies among cases and controls with a Pearson's chi-square test. Odds ratios with the corresponding 95% confidence intervals were also computed. Twins in a pair are not independent and 255 twin pairs are part of the samples from the TwinsUK cohort, to account for nonindependence; the family of origin was included as a random effect in a mixed effects logistic regression model, with affected status being the outcome variable.

Correction for Multiple Testing

The false discovery rate probability method (FDR)[24] was used to adjust for multiple testing for the replication genotypes carried out.

Fixed Effect Meta-analyses

To assess the overall evidence of association, we constructed a Mantel-Haenszel meta-analysis of data from all cohorts. We used the Mantel-Haenszel chi-square test and the Mantel-Haenszel estimate of the odds ratio[25] to provide a summary test and odds ratio. Because twins in a pair are not independent for the TwinsUK sample, the average value of both twins (both affected or both controls) of the 255 twin pairs included was used, so that each of the 255 twin pairs counted as a single individuals for the meta-analysis (Table 1).

Laboratory Methods

Pooled Genotyping

The pools compared consisted of 255 controls and 387 cases affected with either radiographic or clinical OA of the knee (Table 1).

Genomic DNA samples were electrophoresed on 1.5% agarose gels, and samples with intact genomic DNA showing no evidence of contamination by RNA or of DNA degradation as revealed by smearing on agarose-gel electrophoresis were selected for pooling. Intact genomic DNA was diluted to 50 ng/ul concentration on the basis of Quant-iT Picogreen (Invitrogen, Eugene, OR, USA) quantitation, and then concentration was confirmed by repeating the picogreen analysis. Concentrations were adjusted on the basis of these results, and picogreen analysis was repeated. This process was repeated until all samples consistently measured 50 ng/ul. We constructed pools by combining equal volumes of each DNA. All pipetting steps were of volumes greater than 2 ml for minimization of pipetting error. Four replicates from each pool were prepared and hybridized to the Illumina HumanHap550-Duo Genotyping BeadChip microarray (Illumina, San Diego, CA, USA), according to the manufacturer's protocols. The Illumina genotype calling algorithm was modified as previously described,[22] in order to maximize its accuracy in the context of pooled DNA genotyping.

Estimates of allele frequencies were obtained on the basis of the hybridization intensities from the two probes corresponding to each SNP allele. Median signal was determined for both alleles for genotypes AA, AB, and BB for each SNP, enabling us to compute the corresponding relative allele signal, and this was then used to estimate the allele frequency as has been described in detail previously.[22]

Approximately 64,000 SNPs were removed from the list for different reasons: low informativeness among individuals of European descent (MAF $\leq$ 1%), high level of noise, which was determined by comparing allele frequency of the same SNP in the same pool but using data from the different chips (SNPs with a standard deviation of allele frequency above 10%), or because they were in total LD with other SNPs in the list. For some SNPs, when the allele frequency was measured on different chips, some strong outliers were observed and such SNPs were excluded. Because 37 men were part of the controls in the pools but no males were included in the cases, markers from the X chromosome were not considered for analysis. The total number of markers analyzed was therefore 413,461.

Individual Genotyping

All samples except those from the Rotterdam cohort were carried out by Kbioscience, Hertfordshire, UK. SNPs were genotyped with the KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos. Genotyping accuracy, as determined from the genotype concordance between duplicate samples, was 99.6%. The genotyping success rate was 97.9%. All polymorphisms were in Hardy-Weinberg equilibrium in controls (all p > 0.05). Genotyping in the Rotterdam cohort was performed with the Illumina HumanHap 550 SNP array.

Gene-Expression Studies

For gene-expression studies, articular chondrocytes were isolated from femoral cartilage obtained from patients with osteoarthritis undergoing knee-replacement surgery (one female aged 70 years and four males, one aged 66, two aged 73, and one aged 89 years) as described previously.[26] RNA from freshly isolated chondrocytes was obtained with QIAGEN RNAeasy (QIAGEN, Venlo, The Netherlands) kit according to the manufacturer's protocol. After reverse transcription with a RevertAid H Minus complementary DNA (cDNA) synthesis kit (Fermentas, St. Leon-Rot, Germany), cDNA

templates were used for PCR. Primers were designed with Vector NTI (Invitrogen, Carlsbad, CA, USA). The expression of the prostaglandin endoperoxide synthase 2 (*PTGS2* [MIM 600262]) and the phospholipase A2, group IVA (*PLA2G4A* [MIM 600522]) genes was investigated. Primer sequences were *PTGS2* sense primer 5′-AAG TCCCTGAGCATCTACGG-3′, *PTGS2* antisense primer 5′-ATGCCA GTGATAGAGGGTGTT-3′, *PLA2G4A* sense primer 5′-TTGCTGGT CTTTCTGGCTC-3′, and *PLA2G4A* antisense primer 5′-TTCATCAT CACTGTCCGAGC-3′. Complementary DNA was mixed with 0.5 units of Taq polymerase (Eurogentec, Seraing, Belgium), 0.2 mM dNTP, 0.5 M specific primers, and 1.5 mM $MgCl_2$. For *PTGS2* and *PLA2G4A*, annealing temperatures of 55°C and 60°C were used, respectively. PCRs were run for 30 cycles and products were visualized on 1.2% agarose gels.

Allelic Expression Analysis

By using a protocol described previously,[27] we extracted nucleic acid from articular cartilage of UK OA patients who had undergone hip- or knee-replacement surgery (THR and TKR, respectively). The cartilage genomic DNA was used to genotype the patients for SNP rs5275, which is located in the 3′ UTR of *PTGS2*, and for SNP rs4140564, which is located 5′ to *PTGS2*. Three THR (all females) and three TKR (two females and one male) patients were identified as compound heterozygous for both SNPs, whereas one THR (female) and two TKR (one female and one male) patients were identified as heterozygous for SNP rs5275 but homozygous (TT) for SNP rs4140564. The cartilage RNA from these nine patients was then taken forward for an allelic expression analysis using a single base extension (SBE) assay that we have described in detail previously.[28] At least 240 ng of RNA was used for the cDNA synthesis with random hexamers and the SuperScript kit (Invitrogen, Paisley, UK). Two reverse transcription (RT) reactions were performed for each patient: with (+RT) and without (−RT) reverse transcriptase. From each +RT reaction, ≥19 individual PCR amplifications were carried out with forward primer 5′-ACTGTCGATGTTTC CAATGC-3′ and reverse primer 5′-CAAACAAGCTTTTACAGGT G-3′, both located in the 3′ UTR of *PTGS2*. The (−RT) controls did not yield detectable PCR products. The primer 5′-CTAATGTTT GAAATTTTAAAGTACTTTTGGT-3′, which is located immediately adjacent to SNP rs5275, was used for the SBE assay. To ascertain the peak pattern for an assumed 1:1 ratio between alleles, we performed five individual PCR and SBE reactions on the cartilage genomic DNA of each of the nine patients to yield a total of 45 individual genomic DNA measurements. The same PCR primers and SBE primer were used for the cDNA and genomic template. By employing the same analytic conditions for the cDNA and genomic DNA measurements, we were able to use the average of the 45 genomic DNA allelic ratio measurements (representing the assumed 1:1 ratio between alleles) to correct the allelic ratios obtained from the cDNA measurements and thus to account for differences in fluorescent yield and terminator-dye incorporation specific to the assay. Such correction allowed us to obtain exact values of the relative allelic expression of each cDNA measurement. To determine whether there was a significant difference in allelic expression for each patient, we compared the cDNA allelic ratios for that patient to the pooled genomic allelic ratios by using a two-tailed Mann-Whitney exact test.

Results

None of the SNPs tested in the pooled genome-wide scan achieved p values that could be considered as genome-wide significant with a Bonferroni correction for multiple tests i.e., $\log_{10}p = -6.92$ (not shown). We thus selected the genetic variants with a high likelihood of being truly associated with disease by using the false-positive rate probability method (FPRP) developed by Wacholder et al.[23] In total, 28 SNPs resulted from this GWAS that had FPRP ≤ 0.5 (Table 2). These SNPs were individually genotyped in the samples used for the pools and also in an additional 1530 controls and 599 cases from the UK and 272 cases and 258 controls from the U.S. as shown in Figure 1. The 37 male controls in the pools were also individually genotyped, and allele frequencies were found to be the same as in female controls. However, for consistency we did not include data from male samples in any of the subsequent analyses (Table 1).

The 28 SNPs had an average minor allele frequency (MAF) of 13%. Both replication cohorts combined had 80% power to detect as statistically significant odds ratios of 1.39 with p < 0.0017 (Bonferroni α = 0.05 for 28 tests) for MAFs of 13%.

From the individual genotyping of 28 SNPs, we estimated that the genotyping error in pools was 3.1% (SE ± 0.5%), although this estimate cannot be extrapolated to other markers in the pooled GWAS because SNPs with high Z scores had significantly lower errors due to imprecise measurement of the allele frequency in a pool than nonsignificant SNPs (not shown). Combining the test and replication samples, we found that 14 out of the 28 SNPs were nominally statistically significant (p < 0.05); this is consistent with the prior probability of 0.0005 used for the FPRP calculation. However, only one SNP rs4140564 was associated in both the U.S. and UK replication cohorts after adjusting for multiple testing (Table 3).

We selected the seven markers with the smallest overall nominal p values (p < 0.002), and these were then tested for association in a population-based cohort from the Netherlands. Table 4 shows the odds ratios in the Rotterdam cohort for these variants as well as the overall Mantel-Haenszel odds ratio combining all samples, including those used for the GWAS. In addition, a Mantel-Haenszel odds ratio combining all replication samples, excluding those from the pools, is shown.

Only rs4140564 was nominally significantly associated in the Rotterdam cohort with an odds ratio of 1.56 (p < 0.026, Table 3). The summary Mantel-Haenszel odds ratio over all samples was $OR_{MH} = 1.55$ (nominal p < 7 × 10^{-7}) (Table 4). The association of rs4140564 with knee OA in the three replication cohorts, excluding the samples that had been used for the pooled GWAS, was also statistically significant with p < 5.6 × 10^{-6} (Table 4). Because the UK samples used for the GWAS and for replication are derived from three independent studies, we also estimated the odds ratio in each of this individual UK cohorts and found an OR = 1.54 (95% CI 1.05–2.27) for the UK case-control samples, OR = 1.54 (95% CI 1.03–2.31) for the Chingford samples, and OR = 1.56 (95% CI 1.04–2.34) for the TwinsUK samples.

Table 2. SNPs from Pooled GWAS Selected to be Tested in Additional Samples

SNP	Odds Ratio	95%	C.I.	Z Score	$-\log_{10} P$	FPRP with $\pi = 0.0005$	Chr	Position	Nearest Gene(s)
rs4140564	3.34	1.89	5.92	4.312	5.09	0.124	1	184991626	PLA2G4A/PTGS2
rs1539414	1.67	1.19	2.33	4.170	4.82	0.288	1	194475163	DENND1B
rs12041748	2.39	1.59	3.59	4.654	5.79	0.104	1	235550588	RYR2
rs7581129	0.41	0.25	0.66	4.167	4.81	0.498	2	4160033	TSSC1
rs1207421	2.86	1.63	5.02	4.168	4.81	0.460	2	205976100	PARD3B
rs749052	0.28	0.17	0.46	4.305	5.08	0.004	2	232622115	NPPC
rs4955917	1.58	1.25	1.99	4.017	4.53	0.316	3	55021142	CACNA2D3/WNT5A
rs7628387	1.98	1.30	2.78	3.745	4.05	0.227	3	178046256	TBL1XR1
rs1961397	0.50	0.35	0.72	4.352	5.17	0.416	3	189111629	BCL6
rs1990525	3.76	1.93	7.29	4.282	5.03	0.287	4	15097292	C1QTNF7
rs7774801	5.28	2.60	10.89	4.048	4.59	0.029	6	22248420	PRL
rs1324089	2.37	1.49	3.78	4.095	4.68	0.089	6	85022001	C6orf84
rs7757372	1.83	1.18	2.83	4.094	4.67	0.203	6	143972097	PHACTR2
rs2057999	1.81	1.31	2.48	3.986	4.47	0.501	7	21147012	SP4
rs7005969	2.70	1.81	4.04	4.169	4.82	0.213	8	2926103	CSMD1
rs10504460	3.53	2.06	6.05	4.738	5.97	0.018	8	70886578	SLCO5A1
rs2453998	3.85	2.11	7.05	4.826	6.16	0.282	8	104158794	ATP6V1C1
rs2274699	2.47	1.61	3.79	4.182	4.84	0.114	9	71579586	TMEM2
rs7026263	1.77	1.20	2.63	4.114	4.71	0.099	9	133711962	VAV2
rs10823602	3.40	2.02	5.71	4.348	5.16	0.310	10	72145774	ADAMTS14
rs4531428	2.44	1.66	3.60	3.908	4.33	0.028	11	100917249	TRPC6
rs975162	0.50	0.36	0.68	4.785	6.07	0.039	12	125567785	PGBD3P3
rs3818287	1.94	1.35	2.78	4.196	4.87	0.203	14	102500300	CDC42BPB
rs751837	0.42	0.26	0.67	3.913	4.34	0.496	14	102554578	CDC42BPB
rs2896463	0.31	0.18	0.56	4.52	5.51	0.233	14	102560645	CDC42BPB
rs333603	0.69	0.53	0.84	4.008	4.51	0.415	15	64126454	MEGF11
rs7172123	2.59	1.64	4.09	4.552	5.58	0.389	15	66813101	CORO2B
rs6096822	2.83	1.73	4.61	3.957	4.42	0.433	20	35970860	C20orf102

Of the other six SNPs, after excluding the samples in the pools only rs1207421 and rs7628387 remained statistically significant overall (Table 4).

rs4140564 maps to a region in chromosome 1q25 that falls 5′ of both the PTGS2 and the PLA2G4A genes, which encode the cyclooxygenase 2 (COX-2) and the cytosolic phospholipase A2 enzymes, respectively. To assess whether PTGS2 and PLA2G4A were expressed in cartilage, we conducted an RT-PCR analysis for both genes on freshly isolated chondrocytes from knee OA cartilage. Both genes were abundantly expressed in all patients tested (Figure 2). On the other hand, expression of PTGS2 was minimal in placenta and normal synovium, and expression of PLA2G4A was minimal in placenta and was absent in normal synovium.

Analysis of the LD block ($r^2 \geq 0.8$) for individuals of European descent in the Perlegen Genome Browser (build 36) indicated that rs4140564 tags variation covering the whole of the PTGS2 gene, including its 3′ and 5′ untranslated regions SNP (Figure 3), but not the PLA2G4A gene. Because rs4140564 is tagging PTGS2 but only a distant 5′ region of PLA2G4A gene, we hypothesized that this variant, or a variant in LD with it, may affect the expression of PTGS2. To assess whether this was the case, we identified six OA patients (patients 1–6 in Figure 4) who had undergone joint replacement of a hip or of a knee and who were heterozygous both at SNP rs4140564 and at another SNP (rs5275). This second SNP was selected because, un-

like rs4140564, it is part of the PTGS2 RNA transcript (Figure 3). rs5275 is located in the 3′ UTR of PTGS2 and thus enabled us to directly measure allelic expression at PTGS2 by comparing the mRNA allelic ratios of heterozygotes and DNA ratios. For an autosomal locus, DNA ratios for a heterozygote should always be 1.0, but if one of the alleles is in LD with a SNP that affects transcription or mRNA stability, the RNA ratios will be different from 1.0. Two of the six double-heterozygote individuals (patients 3 and 5 in Figure 4) demonstrated highly significant differences in allelic expression ($p < 1.0 \times 10^{-6}$). Patient 3 was a male who had undergone a knee replacement at 76 years of age, whereas patient 5 was a female who had undergone a hip replacement at 65 years of age. SNPs rs4140564 and rs5275 have different minor-allele frequencies and are not in complete LD (pair-wise $r^2 = 0.14$) as seen by the haplotype frequencies in UK samples shown in Figure 4. However, we can conclude that for patients 3 and 5, marker rs4140564 is linked to a regulatory variant of PTGS2 whose alleles mediate different levels of PTGS2 expression.

We also investigated three OA patients (patients 7–9 in Figure 4) who were heterozygous at rs5275 but homozygous (TT) for the marker associated with OA rs4140564. One of these patients (patient 9, a male who had undergone a knee replacement at age 68) demonstrated significant differences in allelic expression ($p < 0.005$).

Table 3. Association of 28 Markers with Knee OA in Two Replication Sets

SNP ID	MAF % Controls/OA	OR	UK (95% CI)	p Value Nominal	p Value FDR	OR	US (95% CI)	p Value nominal	p Value FDR	Discovery + Replication OR$_{MH}$ (95% CI)	p Value Nominal
rs4140564[a]	5.4/8.4	1.44	(1.11–1.87)	0.0056	0.0391	1.77	(1.21–2.57)	0.0026	0.0242	1.59 (1.31–1.94)	3×10^{-6}
rs1539414	21.8/22.5	1.04	(0.89–1.23)	n.s.	n.s.	0.81	(0.61–1.09)	n.s.	n.s.	1.06 (0.94–1.21)	n.s.
rs12041748	12.7/14.6	1.02	(0.84–1.24)	n.s.	n.s.	1.16	(0.88–1.54)	n.s.	n.s.	1.17 (1.01–1.35)	0.04
rs7581129	6.3/6.7	0.92	(0.7–1.22)	n.s.	n.s.	2.30	(1.40–3.77)	0.0007	0.0105	0.99 (0.79–1.25)	n.s.
rs1207421	9.3/12.8	1.40	(1.14–1.72)	0.0012	0.0163	1.04	(0.69–1.57)	n.s.	n.s.	1.46 (1.24–1.73)	6×10^{-6}
rs749052	7.2/7.0	1.11	(0.85–1.44)	n.s.	n.s.	1.20	(0.76–1.89)	n.s.	n.s.	0.93 (0.75–1.15)	n.s.
rs4955917	26.1 /27.6	1.06	(0.92–1.23)	n.s.	n.s.	0.85	(0.61–1.17)	n.s.	n.s.	1.05 (0.95–1.24)	n.s.
rs7628387	12.6/16.0	1.26	(1.05–1.52)	0.0154	0.0862	1.29	(0.90–1.83)	n.s.	n.s.	1.30 (1.12–1.51)	0.0007
rs1961397	10.7/10.1	0.98	(0.78–1.23)	n.s.	n.s.	1.02	(0.69–1.52)	n.s.	n.s.	0.91 (0.76–1.08)	n.s.
rs1990525	7.5/7.5	0.90	(0.7–1.16)	n.s.	n.s.	0.64	(0.40–1.05)	0.0755	n.s.	1.04 (0.85–1.28)	n.s.
rs7774801	9.5/12.7	1.18	(0.95–1.47)	n.s.	n.s.	1.34	(1.00–1.80)	0.0533	n.s.	1.38 (1.18–1.62)	6×10^{-5}
rs1324089	22.1/23.6	1.01	(0.85–1.18)	n.s.	n.s.	0.98	(0.74–1.30)	n.s.	n.s.	1.11 (0.98–1.26)	0.09
rs7757372	22.7/26.6	1.13	(0.97–1.31)	n.s.	n.s.	1.09	(0.82–1.44)	n.s.	n.s.	1.24 (1.09–1.40)	0.0007
rs2057999	16.5/19.1	1.13	(0.95–1.35)	n.s.	n.s.	1.40	(1.02–1.91)	0.0343	n.s.	1.21 (1.05–1.39)	0.006
rs7005969	17.7/18.3	0.89	(0.75–1.05)	n.s.	n.s.	0.89	(0.64–1.24)	n.s.	n.s.	1.05 (0.91–1.20)	n.s.
rs10504460	8.4/10.1	1.05	(0.84–1.32)	n.s.	n.s.	0.96	(0.58, 1.51)	n.s.	n.s.	1.19 (1.00–1.51)	0.050
rs2453998	4.9/5.1	0.89	(0.65–1.22)	n.s.	n.s.	1.00	(0.61–1.64)	n.s.	n.s.	1.05 (0.81–1.36)	n.s.
rs2274699	8.0/8.8	1.04	(0.83–1.31)	n.s.	n.s.	0.71	(0.43–1.17)	n.s.	n.s.	1.11 (0.92–1.34)	n.s.
rs7026263	15.4/16.7	1.02	(0.85–1.22)	n.s.	n.s	1.08	(0.77–1.51)	n.s.	n.s.	1.11 (0.96–1.28)	n.s.
rs10823602	9.7/11.6	0.93	(0.74–1.17)	n.s.	n.s.	1.18	(0.83–1.69)	0.0272	n.s.	1.2 (1.02–1.41)	0.025
rs4531428	18.6/22.0	1.11	(0.94–1.31)	n.s.	n.s.	1.37	(1.02–1.85)	0.0388	n.s.	1.22 (1.07–1.40)	0.003
rs975162	14.2/12.1	0.99	(0.81–1.21)	n.s.	n.s	0.68	(0.50–0.93)	0.0145	0.1014	0.82 (0.70–0.95)	0.009
rs3818287	14.7/15.8	0.99	(0.83–1.19)	n.s.	n.s.	1.09	(0.78–1.54)	n.s.	n.s.	1.09 (0.94–1.27)	n.s.
rs751837	11.6/9.8	0.67	(0.53–0.84)	0.0007	0.0188	1.43	(0.98–2.08)	0.0601	n.s.	0.76 (0.63–0.90)	0.0015
rs2896463	8.7/7.1	0.68	(0.52–0.89)	0.0046	0.0432	1.33	(0.87–2.05)	n.s.	n.s.	0.74 (0.60–0.91)	0.003
rs333603	24.8/24.8	0.83	(0.71–0.98)	0.0238	0.0953	1.69	(1.28–2.22)	0.0002	0.0046	0.95 (0.84–1.08)	n.s.
rs7172123	9.0/12.2	1.31	(1.04–1.64)	0.0204	0.0954	1.34	(1.01–1.77)	0.0400	n.s.	1.37 (1.16–1.62)	0.0001
rs6096822	7.2/8.3	1.01	(0.78–1.29)	n.s.	n.s.	1.27	(0.92–1.77)	n.s.	n.s.	1.17 (0.97–1.41)	0.10

Odds ratios and 95% confidence intervals are shown for each replication study along with the nominal and false discovery rate (FDR) adjusted p values (if ≤0.10). The summary Mantel-Haenszel odds ratio for all samples (discovery plus replication combined) is shown. Minor allele frequencies (MAF) are averaged over the U.S. and UK control and case samples.
[a] SNPs with nominal overall p values < 0.002 are highlighted in bold.

Discussion

By using a pooled genome-wide scan approach and subsequently testing these variants in independent cohorts, we uncovered seven SNPs associated with knee OA but only one that is associated in the five cohorts studied. Two of those seven SNPs, rs7628387 and rs1207421 were overall significantly associated when samples used for the GWAS were excluded. rs1207421 maps to chromosome 2q33, not far from a previously reported linkage peak for hip OA at 2q31.1.[4] Moreover, this SNP maps in the middle of a genome-wide linkage peak of extended early-onset OA families,[29] making it likely that the genomic region near this marker harbors as-yet-unknown OA-susceptibility genes. rs7628387 on the other hand, maps to 3q26, which is not near any known linkage peak.

Table 4. Association of Seven Markers in the Rotterdam Cohort and Meta-analysis in All Study Samples and in Replication Samples Only

SNP ID	O.R. and Nominal p Value in Rotterdam Study		OR$_{MH}$ All	95% CI	Nominal p Value, All Samples	OR$_{MH}$ Replication Samples Only[a]	95% CI	Nominal p Value Replication, Samples Only[a]
rs4140564	1.56	(1.05–2.32), p < 0.026	1.55	(1.30–1.85)	6.9×10^{-7}	1.54	(1.28–1.86)	5.6×10^{-6}
rs1207421	1.14	(0.81–1.61), n.s.	1.40	(1.20– 1.62)	1.2×10^{-5}	1.28	(1.09– 1.50)	0.0028
rs7628387	1.02	(0.64–1.27), n.s.	1.23	(1.08–1.41)	0.0022	1.19	(1.03–1.38)	0.014
rs7774801	0.90	(0.64–1.27), n.s.	1.28	(1.10–1.48)	8.0×10^{-4}	1.15	(0.98–1.34)	0.073
rs7757372	1.03	(0.82–1.30), n.s.	1.19	(1.07–1.32)	0.0019	1.10	(0.98–1.23)	n.s.
rs751837	1.06	(0.76–1.49), n.s.	0.81	(0.69–0.95)	0.0072	0.87	(0.74–1.04)	n.s.
rs7172123	0.76	(0.54–1.07), n.s.	1.22	(1.05–1.42)	0.0070	1.13	(0.99–1.29)	0.077

[a] With data from the UK, U.S., and Rotterdam replication cohorts and excluding all samples used for the pooled GWAS.
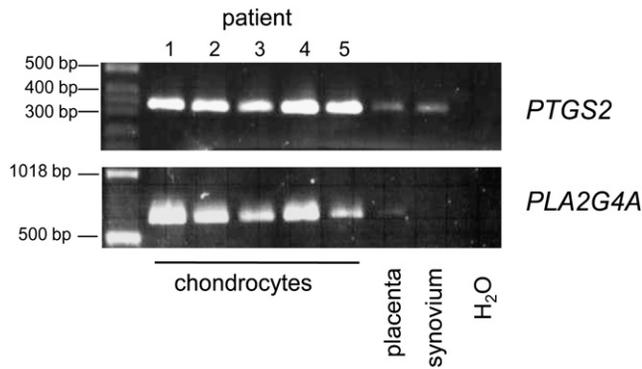
Figure 2. Expression of *PLA2G4A* and *PTGS2* in Chondrocytes
PTGS2 and *PLA2G4A* gene expression was tested by RT-PCR on mRNA obtained from freshly isolated knee-cartilage chondrocytes from five OA patients. The primers amplified a 330 bp *PTGS2* PCR product and a 650 bp *PLA2G4A* PCR product. Human placenta and normal synovium were used as noncartilage control tissues. Water was used as negative control.

The effect sizes derived from the GWAS on a small subset were all overestimated. This pattern can be explained by the so-called "winner's curse," which has been already discussed by other authors,[30] but we note that the odds ratios for predisposing variants in the original GWAS were greater than 1.5 and as high as 5.6, whereas the actual ORs in the combined test plus replication samples were <1.6, consistent with other genetic associations with knee OA reported to date.

The pooled genome-wide scan presented here was underpowered as shown by the fact that no single variant achieved genome-wide significance. A true odds ratio of 2.4 or greater would have been required to achieve 80% power with $\alpha = 1.2 \times 10^{-7}$ in the discovery sample size studied given a MAF that equaled 13% (average for the SNPs in Tables 2 and 3). Nevertheless, the approach utilized illustrates the value of using a pooled GWAS followed by the exclusion of markers that have a high probability of being false positives to uncover variants associated with disease. Several studies showing the validity of pooled

GWAS for complex traits have recently been published, and this approach is considered by some authors as a valid cost-effective method for discovering associations of variants with disease.[31]

The polymorphism that is associated with OA in all samples studied, rs4140564, is located 5′ to *PTGS2*, and we hypothesized that it may be a regulatory polymorphism for this gene or that it may be in linkage disequilibrium (LD) with a regulatory polymorphism. Our allelic expression studies using RNA extracted from the cartilage of OA patients supports the existence of *cis*-acting polymorphisms that can modulate the expression of *PTGS2*. Not all six of the compound-heterozygote patients that we studied however demonstrated differential allelic expression, suggesting that rs4140564 is unlikely to be the regulatory polymorphism itself, but rather it is likely to be in modest LD with a marker-affecting expression. This is further supported by our observation that a patient who was homozygous at rs4140564 also demonstrated differential allelic expression. Identifying the functional regulatory polymorphism will require a comprehensive analysis of other variants within and close to *PTGS2* to see which show the strongest correlation with differential allelic expression. This search may be expedited by an analysis of evolutionarily conserved sequences.

The two genes flanking rs4140564, *PTGS2* and *PLA2G4A*, not only map nearby in the genome but are also both part of the prostaglandin E2 synthesis pathway. Prostaglandin E2 (PGE2) mediates the regulation of important biological processes such as proliferation and differentiation of articular chondrocytes in both normal and pathological states.[32] The production of PGE2 involves a cascade of three enzyme reactions. First, arachidonic acid is liberated from its phospholipid storage sites by a phospholipase A2 (PLA$_2$); it is then acted on by cyclooxygenases and finally by PGE synthase to produce PGE2.[33]

The COX-2 protein is the product of the *PTGS2* gene in humans and has been reported to be expressed in OA meniscus, synovial membrane, and osteophytic fibrocartilage explants and at lower levels in OA articular cartilage, in
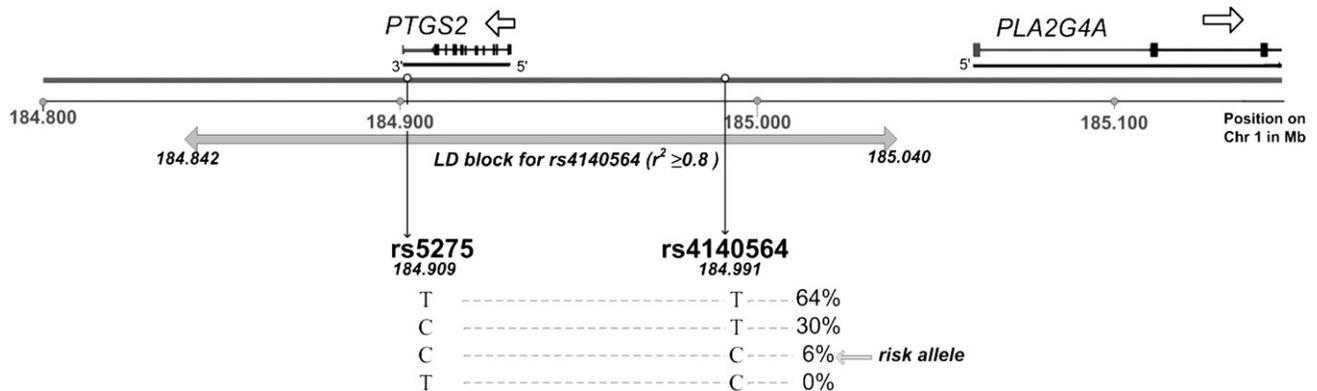

Figure 3. Position on Chromosome 1 of the *PTGS2* and *PLA2G4A* Genes, rs4140564 and rs5275 SNPs
The LD block for rs4140564 (from Perlegen Genome browser build36) is shown, as are the frequencies of rs5275-rs4140564 haplotypes in UK samples.
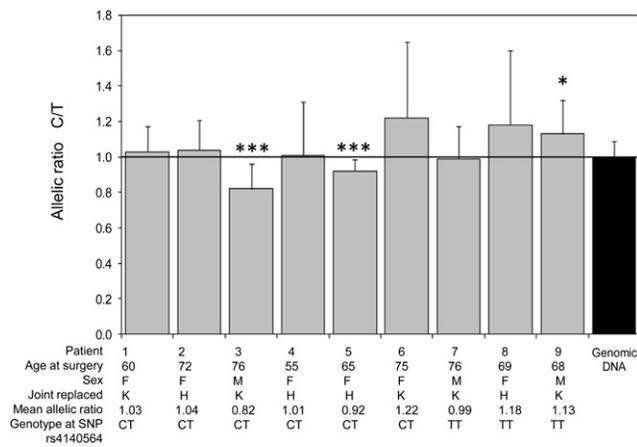
Figure 4. Allelic Expression Analysis at *PTGS2* SNP rs5275

Expression analysis was carried out for nine UK patients who had severe end-stage OA with RNA extracted from articular cartilage. For each patient, 19 (patient 1) or 20 (patients 2–9) individual cDNA amplifications and SBE reactions were performed. Forty-five individual PCR and SBE reactions were performed for genomic DNA (five reactions per patient). The cDNA allelic ratios were compared with the 45 genomic ratios with a two-tailed Mann-Whitney exact test. Four patients had undergone a THR (H), and five patients had undergone TKR (K). Data shown are the mean +SD, *p < 0.005; ***p < 1.0×10^{-6}.

particular during early OA.[34] Our expression-analysis study confirms that the transcripts of two genes related to the synthesis of PGE_2 are abundantly expressed in chondrocytes from OA patients.

Although OA is known to be a noninflammatory pathology, there is a growing body of evidence indicating that there is a significant inflammatory component to disease pathogenesis.[35,36] Strong associations between *PTGS2* variants and radiographic features of OA of the spine have been previously reported,[37] and the results from the present study indicate that genetic variation near *PTGS2* is important also for other forms of OA. Multiple reports have shown that PGE2 is involved in the pathogenesis of OA.[38,39] Increased COX-2 expression has been reported in cartilage and synovial tissues from patients with OA and also in several models of cartilage degradation.[39,40] However, the role of COX-2 in OA is still controversial. Animal studies have shown that during the early phase of inflammation, COX-2 is clearly proinflammatory, but during the later phases of inflammation dominated by mononuclear cells, COX-2 appears to have anti-inflammatory effects by generating an alternate set of anti-inflammatory prostaglandins.[41] Further, whereas some findings suggest that PGE2 mediates the interleukin 1 beta (IL-1β) effect on cartilage degradation,[42] PGE2 may also have positive effects on cartilage by increasing the level of glucocorticoid receptors in chondrocyte cells, influencing cartilage differentiation and proliferation, and mediating the effects of vitamin D on cartilage.[43,44] Recent in vitro studies in human chondrocytes provide evidence that both tumor necrosis alpha (TNF-α) and IL-1β differentially regulate the activation of the apoptotic pathway

in human chondrocyte cells but that PGE2 does not modulate apoptosis.[45] Other studies reported that TNF-α-mediated protection of chondrocytes from nitric-oxide-induced apoptosis requires COX-2 activity.[44]

Given the complex and multiple pathways of regulatory stimuli affecting COX-2 expression, and the discrepant effects that can be observed in vitro, genetic variants that result in either higher or lower expression of COX-2 in response to proinflammatory cytokines or nitric oxide have the potential of resulting in higher risk of OA. This would depend on how transcriptional regulation of the *PTGS2* gene by cytokines and nitric oxide is influenced, on how regulatory loops of the COX enzymes are influenced, and on the stage during the pathogenesis of OA at which the differential response to transcriptional regulatory factors takes place. The data found in our study suggest that an allele in LD with the risk-associated allele at rs4140564 is involved in lower expression of mRNA encoding COX-2. More elaborate experiments are needed to understand the precise mechanism by which rs4140564 or other variants in LD with it are influencing risk of OA.

The expression differences that we observed between *PTGS2* alleles in patients 3, 5, and 9 were relatively small, ranging from 8%–18%. However, these differences are comparable to those observed for the recently reported OA-associated functional SNP rs143383, which is located in the 5′ UTR of the growth/differentiation factor 5 (*GDF5* [MIM 601146]),[26] a gene which has been shown to affect risk of hip and knee OA in both Asian and European individuals.[6] However, because only two of the six rs4140564 heterozygote patients showed decreased *PTGS2* expression, and given the controversial role of COX-2 in OA, we can merely hypothesize that the mechanism involved in increased OA risk could be through differential modulation of *PTGS2* expression. Other possible explanations, which we did not explore, are that rs4140564 or a polymorphism in LD with it might be affecting expression of *PLA2G4A* or that rs4140564 might be in LD with variants affecting the function of either the *PLA2G4A* or *PTGS2* or both gene products.

In conclusion, our data indicate that a polymorphism in LD with variants affecting the expression of the *PTGS2* gene are associated with susceptibility to knee OA in five independent populations and highlight the importance of inflammatory pathways in OA pathogenesis.

Acknowledgments

Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim

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