Testing the druggable endothelial differentiation gene 2 knee osteoarthritis genetic factor for replication in a wide range of sample collections

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ABSTRACT

Objectives: To replicate a previously reported association with osteoarthritis (OA) of the promoter single nucleotide polymorphism (SNP) rs10980705 in the endothelial differentiation gene 2 (EDG2).

Methods: Five collections of samples, four from Europe and one from China, were studied. They included patients with 3 OA phenotypes: 1501 with knee OA, 1497 with hip OA and 376 with generalised OA. A total of 2521 controls were also studied. Allele and genotype frequencies of the rs10980705 SNP were analysed in each individual sample collection and in pooled data. In addition, a meta-analysis to incorporate results from the original Japanese report was performed.

Results: The association of the rs10980705 SNP with knee OA was not replicated in any of the five sample collections studied or in their combined analysis (odds ratio (OR) 1.10, 95% CI 0.98 to 1.22; $p = 0.10$). Meta-analysis of all data, including the original Japanese study, did show association with knee OA (OR 1.15, 95% CI 1.06 to 1.26; $p = 0.002$) but the effect was accounted for by the Japanese data and was less significant than the original report. No association was found with hip OA or with generalised OA.

Conclusions: The original report of a promising genetic association between a druggable G-protein coupled receptor, EDG2, and knee OA has not been replicated. This lack of replication could be due to a modest effect of the promoter polymorphism that will require even larger studies (the winners curse) although a more pronounced effect in the Asian population vs Europeans cannot be excluded.

Osteoarthritis (OA) poses a serious health problem for affected individuals and society.[1] Current prospects are that its burden will increase as no current treatment prevents OA progression. Confronted with this panorama, a recent report has taken advantage of the genetic component of OA to examine G-protein coupled receptor (GPCR) genes in search of promising drug targets.[2] The reasoning behind this study is straightforward. Given that common polymorphisms with modest functional effects are the molecular basis of the OA genetic component, the discovery of a polymorphism that is causally associated with OA indicates that its function is critical for the disease.[3] The choice of GPCR genes expressed in OA is a pragmatic one given that many marketable small-compound drugs are able to inhibit these receptors.[4]

The study was successful with the identification of a single nucleotide polymorphism (SNP), rs10980705, associated with knee OA in Japanese patients following a recessive genetic model. This is a promoter SNP modifying expression of the endothelial differentiation gene 2 (EDG2). It was also shown that EDG2 is the main lysophosphatidic acid (LPA) receptor found in synovium, where this receptor is more abundant than in cartilage. Additionally, it was shown that LPA is able to induce, through EDG2, the expression of inflammatory cytokines interleukin (IL)1β, tumour necrosis factor (TNF)α and IL6, and matrix metalloproteases (MMP)1, MMP3 and MMP13, in a fibroblast-like synovial cell line.[2] Therefore, this study implies that LPA is involved in OA and that its receptor, mainly expressed in synovium, is of relevance too. These are important conceptual advances that require confirmation and study, but the most striking aspect of this report is the prospect of EDG2 as a drug target.

The GPCR family of receptors, also known as seven transmembrane receptors, is the largest family of integral membrane proteins in the human, amounting to about 5% of the human genes.[5] GPCRs mediate signal transmission from the exterior of the cell to its interior. Their dysfunction is involved in many diseases and it is remarkable that up to 45% of all modern drugs act on a GPCR.[4] EDG2 is one of the five receptors for the lipid signalling molecule LPA.[6 7] LPA is generated from cleavage of cellular membrane phospholipids, and it is present at low concentrations in plasma and other body fluids. LPA has not been specifically studied in relation with OA or other arthritis previously to the Mototani *et al* report.[2] In this report, it was shown that the knee OA susceptibility allele at the EDG2 promoter SNP determines increased expression of the receptor. This will contribute to OA through enhancement of the inflammatory response that accompanies and potentiates cartilage destruction.[8] Previous work had already shown that LPA has proinflammation effects on endothelial cells, macrophages and lymphocytes.[9 10]

Herein, we have tried to replicate the genetic association between the rs10980705 SNP and OA by using several collections of samples of diverse phenotype and ethnic origin. This is important because OA genetics shows a remarkable heterogeneity between different phenotypes, gender and

ethnic groups. Previous results were not replicated in the present work, although a weak effect on knee OA could not be excluded.

MATERIAL AND METHODS

DNA samples

Five already described sample collections were used.[11–15] Details of patient and control selection are therefore omitted here. Four collections were from European Caucasians and one from Han Chinese. The European samples were from the Oxford[12] (UK) and Santiago[11] (Spain) collections, including patients with severe primary OA that were undergoing, or had undergone, total knee replacement (TKR) or total hip replacement (THR), controls were 55 years or older and free of OA related complaints; from Thessaly's[13] (Greece) collection with patients with TKR and controls older than 45 years and free of clinical OA; and from the GARP (Genetics, Arthrosis and Progression) study in The Netherlands that recruited sibling pairs concordant for clinical and radiographically confirmed OA at two or more joint sites among hand, spine (cervical or lumbar), knee or hip.[14] Controls for the GARP study were frequency matched to the probands only for age ($\pm$5 years) and geographic region. They were recruited by random sampling of the population by telephone and not further screened for the presence or absence of OA. The Nanjing collection was made of patients with symptomatic and radiographically confirmed knee OA ($\geqslant$2 in the Kellgren–Lawrence grading system) and of controls that had no signs or symptoms of arthritis or other joint diseases.[15] All samples were obtained after the donors had provided their informed consent and with the approval of the respective ethics committees.

Genotyping

The rs10980705 SNP was genotyped in four places: Santiago for the Santiago collection, Leiden for the GARP and Oxford collections, Nanjing for the Nanjing collection and the RIKEN institute at Tokyo for the Thessaly collection. In Santiago and Nanjing a fluorescent 5′ exonuclease assay designed as a TaqMan Genotyping Assay was used (Applied Biosystems, Foster City, California, USA). In Leiden, mass spectrometry (the homogeneous MassARRAY system; Sequenom, San Diego, California, USA), was used following standard protocols. In Tokyo, sequencing of PCR products using the ABI3700 DNA analyser (Applied Biosystems) was used according to the manufacturer's instructions.

Statistical analysis

Concordance with Hardy–Weinberg equilibrium (HWE) was tested in controls from each sample collection. In a first analysis, allelic and genotypic frequencies from each phenotype and sample collection were considered separately. Subsequently, all results were considered with a meta-analysis approach. Two different meta-analyses were conducted, one to test for replication and the other to assess the global evidence for an association by including data from the original Japanese report.[2] Genotype analysis and meta-analysis were performed assuming additive and recessive models because the first is the most common in complex diseases, and because the second best fitted results in the Japanese report.[2] Meta-analysis was performed by pooling odds ratios (OR) from the individual studies, given that the GARP study needs correction of the standard errors for family relationships among the patients with OA. This correction was performed with the robust standard error approach implemented in STATA (StataCorp, College Station, Texas, USA). Meta-analysis was performed with the random effects model given that previous genetic and epidemiological studies have provided evidence that genetic susceptibility differences may exist between OA phenotypes, between the two sexes and between ethnic groups. Heterogeneity of effect size between studies was assessed with the I^2 statistic for inconsistency and the Cochran Q statistic. This later statistic provides a significance value for heterogeneity but lacks sensitivity when analysing a reduced number of studies. The I^2 statistic reflects the percentage of the observed effects that is not explained by chance variation among studies as a percentage.[16] A customised version of Statistica 7.0 (Statsoft, Tulsa Oklahoma, USA) was used for the individual collection data, and R software was used for meta-analysis (http://www.r-project.org/). Statistical power was estimated with the "power and sample size calculations" software.[17]

RESULTS

Genotypes from controls in each of the sample collections were according to HWE. The replication study comprised a total of 3102 patients with OA and 2521 controls. For the meta-analysis, additional 882 patients with knee OA and 919 controls from the original Japanese study were included.[2]

New knee OA data

A total of 1501 patients with knee OA have been analysed. No significant differences were observed in the MAF of the rs10980705 SNP in any of the sample collections (table 1). Only three collections, those from Oxford, Nanjing and Santiago, showed a nominally higher frequency of the A allele in patients, but this change was far from significant. No difference at all could be seen in the samples from Thessaly or the GARP study. Very similar results were observed with genotypes according to an additive genetic model (data not shown). Analyses following a recessive model for the AA genotype at the rs10980705 SNP were also negative (table 1). Therefore, data from the individual sample collections did not replicate the EDG2 association reported in the Japanese. This could be due to lack of power and this was addressed by meta-analysis in a subsequent step. We also checked if these results were affected by considering gender of the subjects. No single group of patients showed significant association either in women or men (supplementary material) although it was near significant in Oxford women ($p = 0.05$) and Thessaly men ($p = 0.05$) for the allelic model (and significant in Thessaly men according to a recessive genetic model, $p = 0.004$, but the number of subjects was too low, 65 cases, to be confident in this result).

Hip OA data

Two collections included patients that required hip joint replacement because of OA (THR) and the GARP collection included subjects that were assessed as having hip OA. In total 1497 patients with hip OA were studied. None of these three collections showed significant differences in the rs10980705 SNP allele frequencies (table 1). There was only a nominal increase in the GARP collection, no change at all in the Oxford collection and a nominal decrease in the Santiago collection. Genotype analyses with either an additive (data not shown) or a recessive model (table 1) did not show any significant differences. Stratification by gender also yielded negative results (supplementary material). In addition, the patients from the GARP

Table 1 Allele and genotype frequencies of the rs10980705 endothelial differentiation gene 2 (EDG2) single nucleotide polymorphism (SNP) in the new collections of samples incorporated in this study

Samples	MAF	OR allele (95% CI)	p_a	Genotypes			OR recessive (95% CI)	p_r
				CC	CT	TT		
Oxford (UK):								
Controls	0.24 (357/1470)			0.57 (416)	0.38 (281)	0.05 (38)		
TKR	0.27 (196/732)	1.14 (0.9 to 1.4)	0.2	0.54 (198)	0.38 (140)	0.08 (28)	1.52 (0.9 to 2.5)	0.1
THR	0.24 (544/2234)	1.00 (0.9 to 1.2)	1.0	0.57 (638)	0.37 (414)	0.06 (65)	1.13 (0.8 to 1.7)	0.6
Leiden (The Netherlands):								
Controls	0.24 (355/1478)			0.58 (428)	0.36 (267)	0.06 (44)		
GARP knee	0.23 (73/318)	0.94 (0.7 to 1.3)	0.7	0.58 (93)	0.37 (59)	0.04 (7)	0.73 (0.3 to 1.7)	0.4
GARP hip	0.27 (60/226)	1.14 (0.8 to 1.6)	0.4	0.53 (60)	0.41 (46)	0.06 (7)	1.04 (0.4 to 2.6)	0.9
GARP	0.24 (178/752)	0.98 (0.8 to 1.2)	0.9	0.58 (219)	0.36 (136)	0.06 (21)	0.93 (0.5 to 1.6)	0.8
Santiago (Spain):								
Controls	0.25 (132/538)			0.58 (157)	0.34 (92)	0.07 (20)		
TKR	0.27 (135/500)	1.14 (0.9 to 1.5)	0.4	0.52 (130)	0.42 (105)	0.06 (15)	0.79 (0.4 to 1.6)	0.5
THR	0.22 (117/534)	0.86 (0.6 to 1.1)	0.3	0.61 (164)	0.33 (89)	0.05 (14)	0.69 (0.3 to 1.4)	0.3
Thessaly (Greece):								
Controls	0.20 (148/758)			0.65 (246)	0.31 (118)	0.04 (15)		
TKR	0.20 (145/720)	1.04 (0.8 to 1.3)	0.8	0.64 (230)	0.32 (115)	0.04 (15)	1.06 (0.5 to 2.2)	0.9
Nanjing (China):								
Controls	0.24 (190/798)			0.59 (236)	0.34 (136)	0.07 (27)		
Knee OA	0.26 (193/732)	1.15 (0.9 to 1.4)	0.2	0.57 (208)	0.34 (123)	0.10 (35)	1.46 (0.9 to 2.5)	0.2

Odds ratios (OR) are presented together with 95% CIs.
GARP, Genetics, Arthrosis and Progression study; MAF, minor allele frequency (number of minor alleles/total alleles); p_a, p value of comparing allele frequencies; OA, osteoarthritis; p_r, p value of the recessive genetic model; THR, total hip replacement; TKR, total knee replacement.

study, who are characterised by generalised OA, did not show any difference in the rs10980705 EDG2 SNP frequencies (table 1).

Meta-analysis for testing replication

We combined data from the five new knee OA collections using a meta-analysis approach. Frequencies of the A allele of the rs10980705 EDG2 SNP showed no significant difference between cases and controls (fig 1) with an OR of 1.10 (95% CI 0.98 to 1.22), p = 0.10. No inconsistency between studies was detected ($I^2 = 0\%$). A very similar result was obtained by considering the OR of the genotype additive models from the combined five knee OA collections (OR 1.10, 95% CI 0.99 to 1.23, p = 0.08 with $I^2 = 0\%$). Meta-analysis of the results according to a recessive genetic model was more clearly negative (OR 1.17, 95% CI 0.88 to 1.56, p = 0.29 with $I^2 = 5.9\%$). Similarly, meta-analyses of allele frequency results stratified by gender did not show significant association neither in women (OR 1.08, 95% CI 0.91 to 1.27, p = 0.4 with $I^2 = 34\%$), nor in men (OR 1.06, 95% CI 0.88 to 1.29, p = 0.5 with $I^2 = 0\%$). Therefore, association between the rs10980705 EDG2 SNP and knee OA was not replicated.

Combined analysis of data relative to hip OA was clearly negative (OR 0.99, 95% CI 0.87 to 1.12, p = 0.85 with $I^2 = 0\%$) for allelic frequencies and similar results were found for the additive and recessive genetic models, and for data stratified by gender (data not shown).

Meta-analysis to assess all available evidence

A global analysis including all of the knee cases from this replication study and from the original Japanese report was subsequently performed. The A allele frequencies were higher in patients with knee OA in this analysis (p = 0.002) with an OR of 1.15 (95% CI 1.06 to 1.26, with $I^2 = 0\%$) that reflects a very modest effect (fig 1). Almost identical results were obtained with an additive genetic model (data not shown). Meta-analysis of data stratified by gender approached significance in women (OR 1.14, 95% CI 0.99 to 1.30, p = 0.063) but not in men (OR 1.11, 95% CI 0.95 to 1.30, p = 0.2), but differences in effect sizes between the two sexes were small. In addition, data from the Japanese samples, which were not stratified by gender when originally reported, did not show a clear difference of effect between women and men (supplementary material).

Analysis of genotypes according to a recessive genetic model showed significant heterogeneity between the different sample collections ($I^2 = 52\%$, $P_Q < 0.05$) and the global result approached significance with this model (OR 1.40, 95% CI 0.99 to 1.98, p = 0.054). Heterogeneity was largely dependant on the resident Japanese collection that showed a minor HWE imbalance in controls. Once a correction for this imbalance was applied,[18] the significant heterogeneity disappeared ($I^2 = 36\%$, $P_Q = 0.2$), and the association became significant with this genetic model (OR 1.36, 95% CI 1.02 to 1.82, p = 0.035).

DISCUSSION

Identification of genetic factors involved in complex diseases such as OA is plagued with difficulties, even if the success stories are becoming more and more common.[3 19] Two of the important limits to progress in this field are the low penetrance of each susceptibility factor and the heterogeneity among patients. A priori, it is likely that these two limits have a relevant role in our study.

With regard to low penetrance, the results reported in Mototani *et al*[2] showed ORs 1.24 to 1.28 in the allele frequency comparisons that represent already a modest effect size. In effect, the limit of sensitivity with commonly achievable sample sizes is often situated at 1.15.[20 21] This is precisely the OR we have observed in our meta-analysis of all available knee OA data. When we aimed to replicate the original finding the OR was even lower, at 1.09. The number of samples required to detect effect sizes like these are >4200 for OR 1.15 and >9200 for OR 1.10 (number of samples = cases+controls at a 1:1 ratio

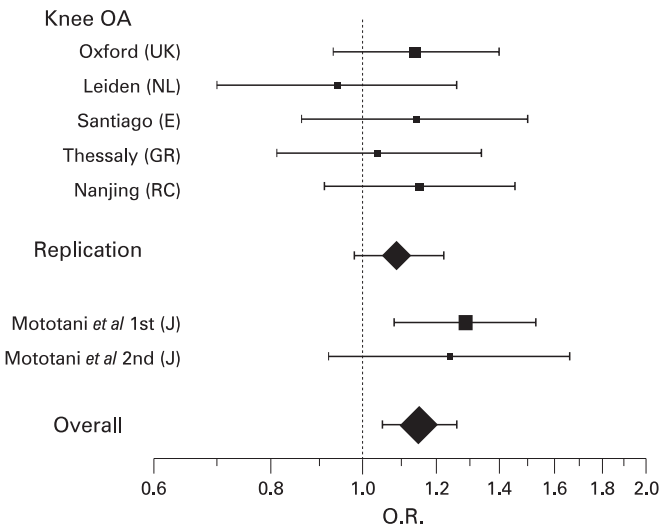

Figure 1 Plot of the odds ratio (OR) and 95% CI for allele frequency comparisons between patients with knee OA and controls in each collection of samples and in meta-analysis of the replication set of samples, and of knee OA overall, after including also the two sets of data from Mototani *et al*[2] (first case: case-control study and second: resident cohort). Size of squares is proportional to study size. Size of rhombi is arbitrary.

for power 0.8 and $\alpha = 0.05$, without misclassification or error). As we have obtained data on 4022 subjects in the replication sample collections, the power of our analysis was enough to detect an effect as strong as that observed in Japanese patients in the absence of heterogeneity (a sample of approximately 2500 cases+controls is large enough for an OR>1.2), but not enough to detect effects below OR 1.15. Therefore, from this analysis it is possible to conclude that the knee OA samples genotyped in our replication study were enough to exclude an effect as strong as that reported by Mototani *et al*[2] but not a smaller one.

The other common source of difficulty in the search of OA genetic factors is heterogeneity. There is abundant evidence of genetic heterogeneity in OA across phenotypes, gender and ethnic groups.[22–25] However in our analysis of knee OA, we did not detect any source of significant heterogeneity either between Asians and Europeans or between women and men. Therefore, heterogeneity is unlikely to be behind our failure to replicate the association found in the Mototani *et al* report.[2] However, to more confidently exclude heterogeneity, particularly ethnic heterogeneity, it will be necessary to have data from more sample collections. At most, we suspect that there is a weak effect of the polymorphism that will need larger sample sizes than those used by us for replication, with the stronger association found in Japanese a reflection of the winner's curse phenomenon.[26] This would imply that the result obtained by Mototani *et al*[2] is probably an overestimation of the true effect size in Japanese because the estimate is conditional on that study being the first to detect association.

We considered it important to use meta-analysis to test the overall evidence for association of the rs10980705 SNP with OA. For this, we took into account all available data including those from the Japanese discovery report. This analysis showed a clear association for knee OA at an allelic and at a genotypic level according to an additive genetic model without evidence of heterogeneity between the studies. Therefore, it is tempting to conclude that the association could be genuine but of low magnitude. However, this result does not represent replication as it was dependent on the original report and therefore the association of EDG2 remains unconfirmed by independent data. There was no evidence of association of the EDG2 SNP with susceptibility to hip OA, in spite of this stratum being adequately powered to detect association. There was also no evidence of association to generalised OA, although this stratum is small and therefore lacks power.

EDG2 is an attractive therapeutic target. It will be possible to validate this target without confirmation of the genetic association, but obtaining genetic evidence will increase the chances of a beneficial effect of EDG2 specific drugs. The progressive availability of larger OA sample collections and of wider collaboration between researchers from different parts of the world, of which our work is a clear testimony, makes it possible that the needed sample sizes will be quickly attainable.[21] EDG2 may well harbour alleles conferring susceptibility for OA, but our analysis using European and Asian cohorts has failed to replicate the previously reported association to SNP rs10980705. The ongoing genome-wide association scans entailing near complete coverage of the nuclear genome in large OA cohorts (21) will enable us to assess definitively the role, if any, that polymorphism at EDG2 has on OA susceptibility.

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