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ABSTRACT

Objectives: Osteoarthritis (OA) is a joint disease common in the elderly. There is a prior functional evidence for different matrix metalloproteinases (MMPs), such as *MMP8* and *MMP9*, having a role in the breakdown of cartilage extracellular matrix in OA. Thus, we analyzed whether the common genetic variants of *MMP8* and *MMP9* contribute to the risk of OA. **Materials and methods**: In total, 13 common tagging single-nucleotide polymorphisms (SNPs) were studied in a discovery knee OA cohort of 185 cases and 895 controls. For validation, two knee OA replication cohorts and two hand OA replication cohorts were studied (altogether 1369 OA cases, 4445 controls in the five cohorts). The χ^2 test for individual study cohorts and Cochran–Mantel–Haenszel test for combined meta-analysis were calculated using Plink. **Results**: The rs1940475 SNP in *MMP8* showed suggestive association in the discovery cohort (OR = 0.721, 95% CI 0.575–0.906; p = 0.005). Other knee and hand OA replication study cohorts showed similar trend for the predisposing allele without reaching statistical significance in independent replication cohorts nor in their meta-analysis (p > 0.05). Meta-analysis of all five hand and knee OA study cohorts yielded a p-value of 0.027 (OR = 0.904, 95% CI 0.826–0.989). **Conclusions**: Initial analysis of the *MMP8* gene showed suggestive association between rs1940475 and knee OA, but the finding did not replicate in other study cohorts, even though the trend for predisposing allele was similar in all five cohorts. MMP-8 is a good biological candidate for OA, but our study did not find common variants with significant association in the gene.

Introduction

The osteoarthritis (OA)-predisposing genes identified so far have been able to explain only little of the 39–65% heritability of this joint-destructing disease (1,2). The disease affects the bone, cartilage, and synovium, inflammatory factors playing a role in the process. One characteristic feature of the cartilage degeneration in OA is the breakdown of extracellular matrix by different matrix metalloproteinases (MMPs) (3). Many studies have shown the role of MMPs in OA, MMP-1 and MMP-13 being some of the most relevant in OA (4,5). Tetlow et al. (6) showed that the expression of different cartilage-breaking MMPs of chondrocytes was greater in the superficial zone of

cartilage where degenerative changes were more common compared to the deeper zones.

The type I, II, and III collagen-breaking MMP-8 seems to play some role in OA, even though it cannot be considered as the main factor in OA. The level of MMP-8 in the cartilage has shown to be higher in OA patients than in controls (p = 0.0135) (7) and the expression of MMP-8 is significantly increased in the joints of mice that have collagen-induced arthritis compared to healthy mice (8). Besides type II collagen, another component of the cartilage is the type IX collagen stabilizing the collagen network. MMP-9 can break down this collagen and other components in the cartilage and has been shown to be differentially

expressed in OA compared to healthy cartilage. For example, MMP-9 was upregulated in human OA cartilage and synovium compared to controls (7). Similarly, Flannelly et al. (9) found the levels of MMP-9 to be higher in the tibial articular chondrocytes of STR/ort mice that spontaneously generate OA than the control CBA mice, and MMP-9 was immunolocalized in growth cartilage. Also, Freemont et al. (10) have shown that the *MMP9* gene is upregulated in deep cartilage layers of human knee OA samples in early disease stages.

Our aim was to investigate if there were common allelic variants in *MMP8* and *MMP9* genes that associate with knee OA. We hypothesized that the studied variants, or variants in high correlation with them, might have an impact on the structure or activity of the enzymes and thus affect their function by protecting or predisposing the carriers to OA.

Study subjects

The study was approved by the ethics committee of the Helsinki metropolitan hospital region in Finland (11,12) and Ethical Committee for Clinical Research of Galicia in Spain. All individuals gave their written informed consent. We analyzed three knee OA study cohorts and two hand OA cohorts (Table 1).

Finnish knee OA discovery cohort

Our knee OA discovery cohort was composed of 185 knee OA cases of Finnish origin. Of these, 110 were

radiologically verified severe (at least grade 3/4 in Kellgren and Lawrance classification), bilateral knee OA cases. Their symptoms were severe enough to fulfill the criteria for knee arthroplasty having pain and walking disability. The symptoms began at a mean age of 52 years (SD 12 years), and the mean age at first arthroplasty was 67 years (SD 8). Study subjects that suffered from RA or had previous severe knee trauma were excluded. Altogether, 28 of the knee OA patients also had physician-diagnosed hand OA. For details, see (11). The other half of the discovery cohort was selected from the population-based Finnish Health 2000 cohort (*n* = 8028). Genome-wide SNP array data were available for 2118 cohort members, who had been ascertained for a nested matched case–control study of metabolic syndrome (the Genmets study) (13). Seventy-five (75) of the 2118 individuals were over the age of 50 years and had a clinician-diagnosed probable or definite knee OA and were included in the discovery knee OA case group. In total, 895 Genmets study individuals were over 50 years of age and did not have clinician-diagnosed probable or definite knee or hip OA and were used as controls. Individuals suffering from RA or having an unknown RA status were excluded from the case and control groups (*n* = 28).

Knee OA replication cohorts

For validation of the initial association, we analyzed one SNP in two independent knee OA replication cohorts. The first replication cohort consisted of 302 clinician-diagnosed knee OA cases and 1700 controls from the Health 2000 study that were not genotyped with the genome-wide SNP array. The cases were selected using the same clinician-diagnosed criteria as in the Finnish knee OA discovery cohort.

The second knee OA replication cohort consisted of 254 knee OA cases and 449 controls of Spanish ancestry. Cases were undergoing knee joint replacement and were included if a rheumatologist considered them to suffer from severe primary OA. Exclusion criteria were inflammatory, infectious, traumatic or congenital joint pathology, and lesions due to crystal deposition or osteonecrosis. Controls were recruited among subjects older than 55 years of age undergoing preoperative work-up for elective surgeries other than joint surgery and who did not show clinical manifestations of OA.

Hand OA replication cohorts

For verification of the finding in another OA type, we used two Finnish hand OA study groups. The first hand

Table 1. Study cohorts.

Study cohort	Cases	Controls
Finnish knee OA discovery cohort	185	895
Radiological*	110	0
Clinical	75	895
Females (%)	72.4	53.6
Finnish knee OA replication cohort	302	1700
Clinical	302	1700
Females (%)	66.2	64.6
Spanish knee OA replication cohort	254	449
Total knee replacement surgery	254	449
Females (%)	80.3	35.4
Knee OA in total	741	3040
Finnish hand OA replication cohort 1*	132	435
Radiological	104	0
Clinical**	28	435
Females (%)	81.8	36.3
Finnish hand OA replication cohort 2	524	970
Visual grading	524	970
Females (%)	67.7	49.5
Hand OA in total	656	1405
All in total	1369	4445

*Family-based study cohort in which 84 individuals are unrelated. Study cohort includes also additional 21 unaffected family member. **In total, 28 study subjects were included in the Finnish knee OA discovery cohort (radiological) and Finnish hand OA replication cohort 1 (clinical). The study subjects were included in the knee OA study group and excluded from hand OA study group in the combined knee and hand OA meta-analysis, and also excluded from the hand OA study group in the independent replication meta-analysis.

OA replication cohort consisted of 132 Finnish radiologically verified severe (at least grade 3/4 in Kellgren and Lawrance classification), bilateral familial hand OA patients and 21 unaffected family members. Eighty-four (84) of the cases were unrelated. Subjects with rheumatoid arthritis (RA) were excluded (11,14). Of these, 28 individuals were also included in the Finnish knee OA discovery cohort. (The 28 study subjects were included in knee OA study group and excluded from hand OA study group in the combined hand and knee OA meta-analysis utilizing all five study cohorts. Also, the 28 study subjects were excluded from the independent replication meta-analysis with four replication cohorts.) The controls (n = 435) were selected from the Finnish twin cohort (males aged 35–70). One twin from a twin pair not having a clinician-diagnosed OA was included in the control group (11).

The second hand OA replication cohort was part of Finnish Helsinki Birth Cohort Study (15). All study subjects were over 56 years of age. Hand joints of the study subjects were visually evaluated and individuals with Heberden's nodes in at least one DIP joint were graded as affected (n = 524). Individuals graded as healthy (n = 970) had visually healthy and symptomless finger joints.

Methods

Genotyping

Genotyping of the finnish knee OA discovery study cohort

MMP8 and MMP9 genes were selected to a tag SNP association study based on their suggestive functional significance in OA and an initial candidate gene analysis of 25 genes (99 SNPs) (12) in a subset of the discovery knee OA study set (data not shown). Tag SNPs of the MMP8 and MMP9 genes also present in the genome-wide Illumina HumanHap 610 chip were selected (Illumina, Inc., San Diego, CA).

In total, 13 SNPs in MMP8 and MMP9 were genotyped in the entire Finnish knee OA discovery cohort using two methods. The Sequenom iPLEX Gold assay on the MassARRAY® Platform (Sequenom Inc., San Diego, CA) was used according to the manufacturer's instructions for genotyping of 110 cases. SNP assays were designed using the MassARRAY™ Assay Design program (Sequenom Inc.) that designed PCR and extension primers and arranged the SNPs in two multiplexes. Each SNP multiplex was validated by genotyping 93 trio samples (i.e., 31 families with two parents and a child). The genotyping was done in 384-well

plates with 35 duplicate samples and 8 non-template wells. The genotype data were assessed using the Sequenom Typer Analyzer program with quality control criteria of at least half of the assays in a sample well performing successfully, visually clear genotype clustering, at least 90% success rate for an assay, and 90% success rate for a sample. If the genotype calling, Mendelian inheritance test using the Pedcheck 1.1 program (16), duplicate samples, or non-template wells revealed unreliable SNP assay, the SNP was excluded from the study.

We also had data available for the selected SNPs from the Illumina HumanHap 610 whole-genome SNP chip (Illumina, Inc.) for 75 cases and 895 controls of the Genmets study (13) that were part of the Finnish knee OA discovery cohort. Markers with genotyping success rate of less than 95%, minor allele frequency less than 1%, and Hardy–Weinberg equilibrium less than 1×10^{-6} were excluded. Of the 2440 individuals with the genome-wide data, we excluded those with genotyping success rate less than 95%, high genomic heterozygosity indicating putative sample contamination, gender discrepancies, or close relatedness with another individual ($\hat{\pi} > 0.1$) (n = 322). Altogether, 2118 individuals and 555,388 markers passed the quality control.

Genotyping of the knee OA replication cohorts

One SNP was genotyped in replication cohorts. The Finnish knee OA replication study cohort was genotyped using the Sequenom system (Sequenom Inc.) as described above.

Spanish knee OA replication study cohort was genotyped using single-base extension reactions with the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA). Genotypes were obtained in the Abi-Prism 3130xl Genetic Analyzer (Applied Biosystems) with a 98.4% call rate.

Genotyping of the hand OA replication cohorts

Finnish hand OA replication cohort 1 was genotyped using homogeneous MassEXTEND™ (hME) technology by the Sequenom MassARRAY® platform (Sequenom Inc.) as recommended by the manufacturer with additional quality control. Genotyping assays were designed using the SpectroDESIGNER 1.3 program (Sequenom Inc.) that designed PCR and extension primers and arranged the SNPs in to multiplexes of one to five SNPs. Each SNP multiplex was validated by genotyping 81 trio samples (i.e., 27 families with two parents and a child) to control for Mendelian incompatibilities using Pedcheck 1.1 program (16). The genotyping was done in 384-well plates with eight duplicate samples and

eight non-template wells. If either automated or manual genotype calling using the Sequenom Typer Analyzer program (Sequenom Inc.), Mendelian inheritance test, duplicate samples, or non-template wells revealed unreliable SNP assay, the SNP was excluded from the study. Further, only SNPs with call rate of over 90% were included.

Finnish hand OA replication cohort 2 was genotyped using the Illumina HumanHap 610 chip (Illumina, Inc.) as described above.

Data analyses

First, the Haploview program (17) was used to measure the linkage disequilibrium (LD) structure of the studied genes in the discovery set controls as r^2 measure. Hardy–Weinberg equilibrium for each SNP was assessed using Haploview (17). The SNPSpd method was used to assess the p-value threshold for 5% significance, considering the LD structure of the studied SNPs. This method utilizes the LD structure of the studied region, and markers in strong LD are considered as one test (http://gump.qimr.edu.au/general/daleN/SNPSpD/) (18,19).

For unrelated individuals, the χ^2 association analysis, meta-analysis (Cochran–Mantel–Haenszel), and heterogeneity between study cohorts in the meta-analysis (I^2 heterogeneity index, Cochran Q) were calculated using the Plink program v1.07 (20) (http://pngu.mgh.harvard.edu/purcell/plink/). For the combined family and case–control setting of hand OA association analysis (Finnish hand OA replication cohort 1), the Pseudomarker program was used. The Pseudomarker program utilizes combined data of families and singletons and calculates the association with the presence of linkage (21,22).

Results

Altogether, 13 SNPs were studied in the Finnish knee OA discovery study cohort. The SNPs covered 62% of the common variation in the *MMP8* gene (±5 kb) and 67% in the *MMP9* gene (±5 kb) according to the Tagger program (http://www.broadinstitute.org/mpg/tagger/) (23). Maximum r^2 correlation of 80% between the markers and allele frequency threshold of 10% was used. Reference population was HapMap CEU: Utah residents with ancestry from northern and western Europe; phased HapMap Release 21 (24). The LD structures of the studied genes in discovery cohort controls are shown in Figure 1. Based on the SNPSpD method (18,19) the level of 5% significance was reached with a p-value of 0.0057 corresponding to the effective number of nine SNPs. The most significant association was further analyzed in independent replication cohorts of knee OA and hand OA.

Two SNPs, rs1940475 and rs3765620, showed an association with knee OA in the Finnish knee OA discovery cohort ($p = 0.005$ and $p = 0.004$, respectively) (Table 2). The two SNPs were in LD with each other (r^2 82%). Due to the high correlation between the SNPs, only the rs1940475 marker was further analyzed in two independent knee OA and in two independent hand OA replication cohorts. In all four study cohorts, the allele frequency difference between cases and controls was in the same direction as observed in the initial discovery study set of 185 cases and 895 controls. However, the initial finding did not replicate statistically significantly in any of the four independent replication study sets of knee and hand OA ($p > 0.05$) nor in a meta-analysis combing the four replication study sets ($p = 0.236$). When including the discovery study set in the meta-analysis,
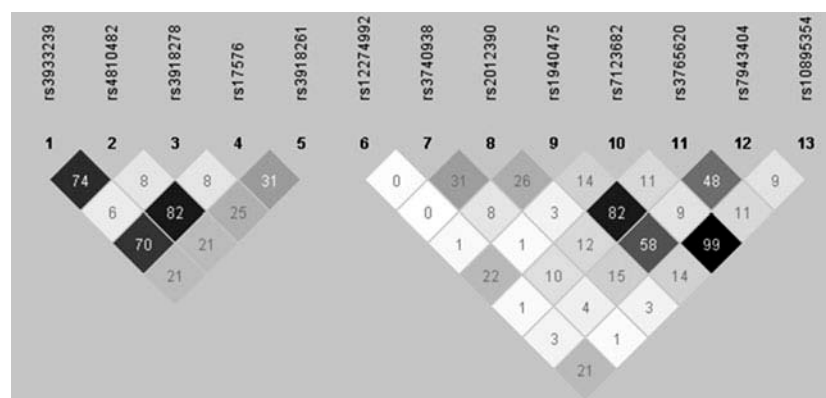

Figure 1. The LD structure of the studied genes in the controls. The LD structure of the *MMP9* and *MMP8* genes is presented as a measure of r^2. Stronger color indicates higher level of pairwise LD between each SNP pair, black color indicating 100% LD.

Table 2. The results of association analysis in the Finnish knee OA discovery cohort.

Chr	Gene	SNP	Location	Al.	Freq ctrls	Freq cases	χ^2 p-value	OR	L95	U95
20	MMP9	rs3933239	44062075	A	0.465	0.473	0.803	1.029	0.821	1.290
20	MMP9	rs4810482	44067957	C	0.396	0.398	0.936	1.009	0.802	1.271
20	MMP9	rs3918278	44069061	A	0.050	0.038	0.328	0.751	0.423	1.335
20	MMP9	rs17576	44073632	G	0.390	0.392	0.945	1.008	0.799	1.272
20	MMP9	rs3918261	44076999	G	0.169	0.175	0.792	1.041	0.772	1.403
11	MMP8	rs12274992	102084124	T	0.051	0.074	0.088	1.470	0.942	2.293
11	MMP8	rs3740938	102092272	A	0.074	0.068	0.688	0.913	0.587	1.422
11	MMP8	rs2012390	102095987	C	0.205	0.186	0.417	0.888	0.666	1.183
11	MMP8	rs1940475[a]	102098458	T	0.493	0.413	0.005	0.721	0.575	0.906
11	MMP8	rs7123682	102099237	C	0.129	0.159	0.132	1.271	0.930	1.738
11	MMP8	rs3765620[b]	102100702	G	0.446	0.363	0.004	0.710	0.562	0.895
11	MMP8	rs7943404	102103785	G	0.378	0.431	0.060	1.245	0.990	1.565
11	MMP8	rs10895354	102104264	C	0.129	0.159	0.125	1.277	0.934	1.746

Chr = chromosome; Location = genomic location in base pairs; Al. = minor allele for which the ORs are given; Freq ctrls = allele frequency in controls; Freq cases = allele frequency in cases; OR = odds ratio; L95 and U95 = 95% confidence interval; [a]Genotyped also in other study cohorts (Table 3); [b]p-value in the Finnish hand OA replication cohort 2 = 0.741 based on the GWAS data; OR = 0.975, 95% CI 0.837–1.135.

Table 3. Meta-analysis of rs1940475.

	Freq ctrls	Freq cases	χ^2 p-value	OR	L95	U95
Finnish knee OA discovery cohort	0.493	0.413	0.005	0.721	0.575	0.906
Finnish knee OA replication cohort	0.473	0.454	0.371	0.924	0.776	1.099
Spanish knee OA replication cohort	0.462	0.461	0.966	0.995	0.800	1.239
Knee OA META			0.039	0.884	0.787	0.994
Finnish hand OA replication cohort 1	0.484	0.428	0.187[a]	0.798	0.571	1.116
Finnish hand OA replication cohort 2[b]	0.474	0.465	0.639	0.965	0.830	1.121
Hand OA META			0.332	0.934	0.814	1.072
Independent replication OA META[c]			0.236	0.943	0.855	1.039
All cohorts OA META			0.027	0.904	0.826	0.989

[a]Only unrelated individuals, 84 cases and 435 controls, were included in the χ^2 analysis. In the family-based association test with 132 affected, 21 unaffected and 435 controls, the p-value was 0.192 using Pseudomarker. The p-value for linkage was 0.0399 in the Pseudomarker analysis; [b]including the 28 clinical hand OA cases that are also part of the Finnish knee OA discovery cohort; [c]excluding the 28 clinical hand OA cases that are also part of the Finnish knee OA discovery cohort.
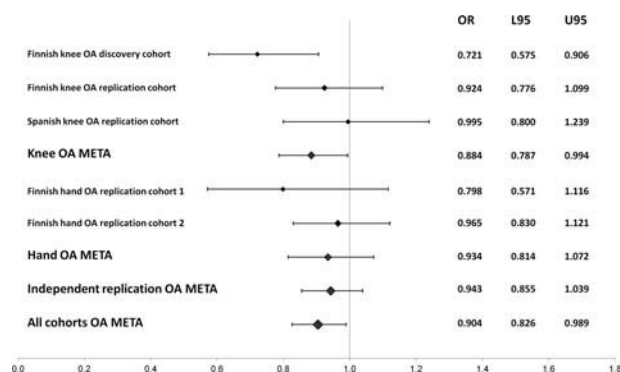

Figure 2. Forest plot of meta-analysis for the T allele of the 1940475 SNP. Odds ratios and 95% confidence intervals. The sizes of the dots are in proportion with the amount of cases in the study cohort. Finnish hand OA replication cohort 1 includes the 28 clinical hand OA cases that are also part of the Finnish knee OA discovery cohort; independent replication meta-analysis excludes the 28 clinical hand OA cases that are also part of the Finnish knee OA discovery cohort.

p-value of 0.027 was obtained (Table 3; Figure 2). The I^2 heterogeneity index was 34.9 (1–100) and the p-value for Cochran Q statistic was 0.189, indicating that there was no significant heterogeneity among the five study populations.

Discussion

Altogether, 13 SNPs in the *MMP8* and *MMP9* genes were studied in knee OA, and a statistically significant association with two SNPs (rs1940475 and rs3765620) in high LD with each other in *MMP8* was observed (p < 0.0057). One of the SNPs rs1940475 was further analyzed in four independent replication cohorts, but the finding did not replicate in any of the knee and hand OA cohorts independently nor in a combined meta-analysis of the independent cohorts, although the trend for the C-allele being more common in OA cases was similar in all five study cohorts.

The power to detect the level of association seen for rs1940475 was 50% in the Finnish knee OA discovery cohort. Roughly 500 cases would have been needed for 80% power of detecting the association with 5% confidence level corresponding 95% CI. When assuming similar allele frequencies for the follow-up cohorts as in the discovery cohort, the power to detect association in the individual follow-up cohorts varied between 30 and 80%. The power was 99.9% in the meta-analysis of the four independent replication cohorts. However, the effect size in the Finnish knee OA discovery cohort has probably been overestimated (25). This is in accordance with previous publications showing that

effect sizes typically seen in complex disease mapping require thousands of study samples for detection of association or for excluding the role of a single variant in a disease (26).

Both originally associated SNPs are non-synonymous. The rs1940475 changes the positively charged lysine to negatively charged glutamic acid (Lys87 to Glu) and the rs3765620 changes the polar threonine to nonpolar isoleucine (Thr32 to Ile). Both amino acid changes were predicted to be tolerated according to the SIFT program (27,28).

We did not observe significant evidence of association to the studied common variants in the *MMP8* gene. There is some functional evidence for the role of MMP-8 in OA, making it an interesting study target, even though it is not the main MMP released in OA. MMP-8 can cleave the major collagen of the cartilage, collagen type II, and also collagens type I, III, other ECM compounds, and non-structural molecules as well and affect neutrophil migration (29). MMP-8 is expressed by neutrophils (30), chondrocytes (31–33) especially mature chondrocytes (34), and chondrocytes following a proinflammatory stimulus (35). Billinghurst et al. (3) showed that the increased expression of MMP-8 is associated with increased cleavage of type II collagen in human osteoarthritic cartilage. The MMP-8 mRNA has been shown to be absent from normal cartilage but present in naturally occurring (7,36,37) and in IL-1β-induced OA cartilage similarly to the MMP-8 protein (6,7). MMP-8 mRNA levels were higher in chondrocytes obtained from the surrounding areas of OA lesion when compared with those seen in chondrocytes from other areas of the cartilage (38). Also the MMP-8 protein levels were higher in the superficial zone of OA cartilage and they were especially associated with areas suffering from matrix depletion, fibrillations, chondrocyte clusters, and loss of metachromasia. Protein levels were lower or absent from the deep zone and showed normal hyaline cartilage and chondrocyte morphology (6). Moreover, MMP-8 inhibition has shown promising results. Tetracycline (39), doxycycline (38), and Ro 32-3555 (an orally active collagenase selective inhibitor, Trocade®) (40) seem to inhibit neutrophil MMP-8 activity *in vitro*. Doxycycline has been tested successfully in reactive arthritis patients (41) and also Ro 32-3555 leads to significant protection of cartilage, inhibition of osteophyte formation, and reduction in joint space narrowing in the STR/Ort mouse model of OA (42).

Previous genome-wide association studies (GWASs) have revealed genome-wide significant ($p < 5 \times 10^{-8}$) findings in OA. Many of the findings are performed in large study samples, but as is typical in complex diseases, the effect sizes have been small and fine-mapping is required to identify the causal variants. The previous GWAS findings in OA include extracellular signaling molecule *GDF5* (growth differentiation factor 5) (43,44), *DVWA* (von Willebrand factor) (45), 7q22 region with *GPR22* (G-protein coupled receptor 22) (46,47), a skeletal and pain-related gene *MCF2L* (MCF.2 cell line derived transforming sequence-like, encoding the rho-specific guanine nucleotide exchange factor) (48), the *FTO* gene (fat mass and obesity associated) (48), membrane protein *ASTN2* (astrotactin 2) (49), a wnt signaling gene *DOT1L* (DOT1-like, histone H3 methyltransferase) (50), nuclear receptor activator gene *NCOA3* (nuclear receptor coactivator 3) (51), and *HLA* class II/III region (52).

The arcOGEN Consortium (49) published the details of their top 129 SNP findings ($p < 10^{-5}$) in OA with 7410 OA cases and 11009 controls, but the *MMP8* and *MMP9* genes were not among the published regions nor in the genome-wide significant regions of other GWASs mentioned above. However, the *MMP9* gene is located in the chromosome 20q13.12 locus together with the *NCOA3* gene associating with hip OA in the previous GWAS ($p = 7.9 \times 10^{-9}$, OR = 1.28, 95% CI 1.18–1.39, 11,277 hip OA cases, 78,000 participants) (51), but the two genes are more than 1 Mb apart, thus the negative finding of the current study is likely an independent signal from the association finding of the hip OA GWAS study in another study population.

Conclusions

A suggestive association in a discovery knee OA study cohort was observed with two common SNPs, rs1940475 and rs3765620, in the *MMP8* gene, which has previously been shown to have a functional role in OA. However, the finding was not replicated with statistical significance in follow-up analyses of hand and knee OA.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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