EXTENDED REPORT

Further evidence of the role of frizzled-related protein gene polymorphisms in osteoarthritis

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Objective: To replicate the association of frizzled-related protein (FRZB) non-synonymous polymorphisms with osteoarthritis (OA) susceptibility.

Methods: Three groups of Spanish patients with OA were included: with total joint replacement due to primary OA in the hip (n = 310), or the knee (n = 277), or with hand OA (n = 242). Controls were more than 55 years old and did not show OA (n = 294). SNPs rs288326 (R200W) and rs7775 (R324G) were genotyped.

Results: There were no significant differences in allele frequencies between controls and each of the three groups of OA patients. However, allele G of the R324G SNP showed a trend to be more frequent in patients with a clinical OA syndrome at multiple joints (p = 0.07), specifically in women of the total hip replacement group (8.3% in patients without other affected joints, 13.1% with one, 15.9% with two and 24.1% with more than two additional joints, p for trend = 0.008).

Conclusions: No direct replication of previous OA association findings was obtained but the results suggest that the R324G SNP of the FRZB gene may have an effect in OA development in multiple joints, with a specific severe involvement of the hip in women. This phenotype could reconcile previous studies that showed association either with generalised OA or with hip OA in women.

It is well known that osteoarthritis (OA) is the commonest form of joint disease and a leading cause of disability in older people,¹ but while some people develop OA changes in their 40s and 50s, others enjoy long lives without joint problems. Several factors play a role in the variable OA risk, including age, sex, genetics, ethnicity, behavioural influences, obesity and occupation.² Genetic factors have been shown to account for between 40% and 85%, depending on the phenotype considered, of the variance in liability to OA and they contribute to a twofold to fivefold increase in OA risk in patient siblings.³ However, each genetic factor has a modest effect and, consequently, their identification and study are difficult.^{2–4} A further complication of research in OA derives from a variety of disease phenotypes that seem to depend on different susceptibility factors^{2–4} and could be related to different pathogenic mechanisms.⁵ Therefore genetic variants predisposing to OA in one joint do not have, in general, the same effect in other joints and may even differ between men and women.⁶ Our current knowledge of the disease is too crude to explain these differential effects, or to identify differences between these joint-specific factors and others that seem to predispose to OA more broadly. In spite of these difficulties, several genetic factors have been shown reproducibly to participate in OA predisposition, such as polymorphisms in the oestrogen receptor a gene (ESR1) or the interleukin 1 (IL1) cluster and a repeat polymorphism in the asporin gene.^{2–7}

A series of recent studies show some evidence implicating non-synonymous single nucleotide polymorphism (nsSNPs) of the frizzled-related protein (FRZB) gene, coding for the secreted frizzled-related protein 3 (SFRP3), in a couple of OA phenotypes. The first evidence was obtained by linkage analysis of UK sibling pairs concordant for total hip replacement (THR) for primary OA.⁸ FRZB is in the linked locus, in chromosome 2q, and is a likely candidate because, as in its role of inhibitor of WNT signalling, it is involved in bone and joint development.^{9–11} Association to a nsSNP (rs7775 causing a R324G substitution) in FRZB was found in female probands. Replication of the association in the THR female subset of a case-controlled UK

cohort in the same report strengthened the case for a direct role of the R324G SNP of FRZB in severe hip OA in women.⁸ There was also evidence of a possible effect of another FRZB nsSNP (rs288326 causing a R200W substitution) only in women bearing both the G allele of R324G and the W allele of R200W. Functional experiments showed that the 324G allele of SFRP3 is less effective in blocking WNT signalling, an effect that will be related to a decreased sequestration of WNT ligands in the extracellular space. Additional experiments showed that other SNPs in the FRZB promoter do not significantly affect transcription levels and are unlikely to be alternative causal variants.¹² A subsequent study has obtained confirmatory evidence for the role of the FRZB nsSNPs in radiographic hip OA characterised by severe joint space narrowing in women.¹³ In this case the effect was mainly associated with the R200W SNP, although carriage of both the R200W and R324G rare alleles was a risk factor. Another study showed an effect of the R200W SNP, but not of the R324G SNP, in the difference between women with THR and women with osteoporosis but not in the comparison with healthy controls.¹⁴ Neither of these two replication studies included patients with OA in other joints or men with hip OA. A broader range of OA phenotypes was explored in a third replication study that included both male and female subjects with radiographic OA in the hip or in multiple joint regions in a population-based cohort.¹⁵ They also included sibling pairs selected for the presence of symptomatic OA at multiple sites. In this study the OA predisposition effect of the FRZB nsSNPs was only evident for the R324G SNP and in patients with polyarticular OA (no sex effect was mentioned) but not in those with hip OA. Therefore, it seems that there is already convincing evidence, though not completely conclusive, of the involvement in OA of nsSNPs in the FRZB gene. However, there are still many unresolved questions including whether they play a role only in women, only in hip OA, or in polyarticular OA, or if each of the two non-synonymous SNPs

Abbreviations: FRZB, frizzled-related protein; HOA, hand osteoarthritis; OA, osteoarthritis; THR, total hip replacement; TKR, total knee replacement

has an effect on their own or whether both rare alleles need to be present in the same subject. We have tried to address some of these outstanding questions.

MATERIAL AND METHODS

Patients and controls

Selection criteria and patient characteristics have been described.¹⁶ In brief, subjects were recruited from patients undergoing total hip replacement (THR) or total knee replacement (TKR) only if a rheumatologist considered them to suffer from severe primary OA (a directed questionnaire, the clinical history and radiographs previous to surgery were reviewed). A third group included patients attending the rheumatology unit because of hand OA (HOA) complaints who fulfilled American College of Rheumatology classification criteria.¹⁷ The presence of chronic pain and incapacity in other joint regions different from the one motivating recruitment of the patients, as well as hand deformities in THR and TKR patients, were recorded. These manifestations were considered as evidence of clinical OA by a rheumatologist after excluding other causes by means of anamnesis following a directed questionnaire and review of the clinical history. Radiographs of the additional joint regions were not a requisite.

Controls were older than 55 years. Only the 31.6% who were free of clinical manifestations compatible with OA (radiographic examination was not performed) were included. This selection introduced a bias in the controls: of the 929 voluntary donors 58.2% were women, but after exclusion of those with features compatible with OA only 39.1% were women. Accordingly, comparisons were stratified by sex. The final numbers in each group are shown in table 1. This study was approved by the Ethical Committee for Clinical Research of Galicia and all participants gave their written informed consent. All were of Spanish origin and resided in the reference area of the hospital.

Genotyping

Peripheral blood samples were used to obtain DNA. TaqMan (Applied Biosystems, Foster City, CA) 5' nuclease assays were used to genotype two non-synonymous FRZB SNPs: rs288326 C/T that causes the R200W substitution in exon 4 and rs7775 C/G producing the R324G substitution in exon 6. Primers and

fluorescence-labelled probes were designed and synthesised by Applied Biosystems and their sequences are available from the authors. TaqMan reactions were performed in a total volume of 10 µl containing 24 ng genomic DNA following the Applied Biosystems protocol. A Chromo4 real-time PCR system (MJ Research, Waltham, MA) was used to run these assays. Several samples with different genotypes were sequenced with the Big Dye Ready Reaction Kit (Applied Biosystem) to test the accuracy of the genotypes.

Statistical analysis

Statistical analysis was carried out with the Statistica software (Statsoft, Tulsa OK). Allele frequencies, odds ratios (OR) and their 95% confidence intervals were calculated. Comparison of allele frequencies was done using a two-by-two contingency table with a χ^2 test. Evidence of a gene dose effect was evaluated with univariate logistic regression applying an additive genetic model (codes were: 0 for AA, 1 for Aa and 2 for aa genotypes). Multivariate logistic regression was used to evaluate the effect of the two nsSNPs together. The effect of genotypes in number of OA localisations was determined via logistic regression. Linkage disequilibrium, estimation of haplotype frequencies and comparison of those frequencies between patients and controls were made with the Haploview software.

RESULTS

Three groups of patients have been included and their characteristics are shown in table 1. We have included in this table the percentages of patients showing clinical manifestations compatible with OA affectation in multiple joint regions. Controls were older than 55 years and selected to be free of symptomatic OA. Numbers of men in the TKR and HOA groups were small and results relative to them should be taken with caution. Power to detect an effect with OR = 2.0 in the THR patients was over 0.98, and for an OR = 1.5 it was 0.65 for the R324G SNP and 0.75 for the R200W SNP. Valid genotypes for the two FRZB nsSNPs were obtained in 98.7% of the subjects. Their frequencies were in accordance with the Hardy-Weinberg equilibrium.

In contrast with what could be expected based on some previous reports,^{8, 13} the frequencies of the rare alleles of the R324G SNP and of the R200W SNP were not significantly elevated in women with hip OA compared with healthy controls (table 2). In addition, women with the other two OA phenotypes, TKR and HOA that were studied for the first time

Table 1 Characteristics of patients and controls included in the different groups

Characteristics	OA patients			Controls
	THR	TKR	HOA	
Number of subjects	310	277	242	294
Sex (% women, women/total)	60.6 188/310	80.5 223/277	88.4 214/242	39.1 115/294
Age of disease onset (median IQR*)	61 55–67	56 50–63	50 44–56	
Age at recruitment (median IQR)	69 64–73	69 64–72	61 54–68	68 61–75
BMI (median IQR)	30.1 27.7–	32.9 29.9–	28.3 26.0–	
	32.9	36.0	31.3	
OA in other joint regions % (number)				
No other region	42.6 (132)	35.0 (97)	44.2 (107)	
1 region	31.3 (97)	31.8 (88)	30.2 (73)	
2 regions	16.1 (50)	22.4 (62)	14.5 (35)	
>2 regions	10.0 (31)	10.8 (30)	11.1 (27)	

THR, total hip replacement; TKR, total knee replacement; HOA, hand osteoarthritis.

*IQR, Interquartile range.

Table 2 Minor allele frequencies* of the two nsSNPs of FRZB

	Women	Men	Total
R324G			
Controls	11.6 (26/224)	10.7 (38/354)	11.1 (64/578)
THR	14.1 (52/368)	9.7 (23/236)	12.4 (75/604)
TKR	11.0 (49/444)	12 (13/104)	11.3 (62/548)
HOA	12.1 (51/420)	9 (5/54)	11.8 (58/474)
R200W			
Controls	11.3 (26/230)	17.6† (63/358)	15.1 (89/588)
THR	9.9 (37/372)	14.0 (34/242)	11.6 (71/614)
TKR	13.5 (59/436)	16 (17/106)	14.0 (76/542)
HOA	11.8 (50/422)	9 (5/54)	11.5 (55/476)

*Frequencies expressed as % and number of minor alleles/total number of alleles.

†This frequency was significantly larger than in female controls ($p=0.03$). However, 126 additional male controls showed a W allele frequency (11.5%) very similar to the female frequency.

THR, total hip replacement; TKR, total knee replacement; HOA, hand osteoarthritis.

in relation with these SNPs, did not show differences from controls. In the same way, there were not differences between male patients from any of the three patient groups and male controls (as mentioned male numbers in the TKR and, specially, in the HOA groups were small). Similar negative results were observed in the genotype analysis of the two nsSNPs using an additive genetic model (not shown).

A previous report has indicated that the G allele of the R324G SNP is associated with polyarticular OA.¹⁵ We have found some supportive evidence by comparing the frequencies of this SNP in patients with manifestations compatible with OA in additional joint regions. Notably, the strength of the association increased with the number of joint regions involved. There was a near significant trend when men and women from the three patient groups were considered together, but the relationship became clear after stratifying by groups of patients (table 3). The effect was restricted to patients of the THR group, with no differences in the other two groups: TKR and HOA. Further stratification by sex showed that the association was exclusively observed in women (table 4). Women in the TKR and the HOA groups did not show this association. Frequencies of the R200W SNP, on the other hand, did not show any relationship with the number of additional joint regions with OA-compatible affection (data not shown).

Finally, there was some previous evidence of a collective effect of the rare alleles of the two nsSNPs in OA susceptibility.^{8, 13} The two FRZB SNPs were not in linkage disequilibrium ($D' = 0.07$, $r^2 = 0.003$) in our samples as in all the previously studied populations. The haplotype frequency distribution was not different between THR women and controls, or between the other groups of patients and controls (not shown). In addition, there was not any evidence of a collective effect of the two SNPs in the number of additional joint regions involved. However, it should be noted that the rare alleles of the two nsSNPs appear together in very few subjects, given their low frequency and lack of linkage disequilibrium, and this study lacks power to detect a moderate effect of such a rare event.

Table 3 Minor allele frequencies* of the two nsSNPs of FRZB in patients with OA stratified by number of joint regions in addition to the one that had determined patient recruitment showing OA compatible features

	Number of additionally affected joint regions				p for trend
	0	1	2	>2	
R324G					
All groups	10.1 67/662	12.2 62/506	13.4 39/290	14.5 25/172	0.05
THR	9.7 25/258	12.2 23/188	14.3 14/98	22 13/60	0.01
TKR	10.3 20/194	13.4 23/172	11.3 14/124	9 5/58	0.9
HOA	10.5 22/210	11.0 16/146	16 11/68	13 7/54	0.3
R200W					
All groups	12.9 86/666	11.8 60/508	11.1 32/288	13.8 24/174	0.9
THR	13.0 34/262	8.8 17/192	10.0 10/100	17 10/60	0.9
TKR	16.7 32/192	12.9 22/170	12.5 15/120	12 7/60	0.2
HOA	9.4 20/212	14.4 21/145	10 7/68	13 7/54	0.5

*Frequencies were expressed as % and number of minor alleles/total number of alleles.

THR, total hip replacement; TKR, total knee replacement; HOA, hand osteoarthritis.

Table 4 Minor allele frequencies* of the R324G SNP in patients of the THR group stratified by sex

	Number of additionally affected joint regions				p for trend
	0	1	2	>2	
Women	8.3 8/96	13.1 17/130	16 14/88	24 13/54	0.008
Men	10.5 17/162	10 6/58	0 0/10	0	0.3

*Frequencies were expressed as % and number of minor alleles/total number of alleles.

DISCUSSION

Progressively, new genetic factors that seem implicated in OA susceptibility have been reported.^{2, 4} Evidence involving several of them starts to be compelling though it is not yet clear if any has reached a level of confirmation that could be taken as definitive. Experience in the study of other complex diseases, where rarely a genetic variant is consistently found to have the same effect in different studies, has led some authors¹⁸ to think that only very significant and straightforward results, not involving analysis of subphenotypes or subgroups of patients, should be considered as evidence of a true genetic effect. However, research into OA has been providing examples of variants that show association in successive studies in spite of modest p values and of being restricted to a limited phenotype in the wide spectrum of the disease. In the case of the FRZB gene, some evidence of an effect of two nsSNPs in OA susceptibility has already been found in the four studies that have been conducted.^{8, 13-15} In each case the effect has been modest at best, with p values barely below 0.05, and always restricted to a subgroup of patients. In addition, the evidence of association has not been identical but with particular aspects in each case. Our results should be considered within this framework.

The nsSNP that we have found associated, R324G, has been the susceptibility SNP in two of the previous studies,^{8, 15} and is also the SNP that has been shown to have a functional effect.⁸ This makes it likely that it will be a genuine OA susceptibility variant. In contrast, the R200W SNP was associated with OA susceptibility only in two studies^{13, 14} and no functional consequences have been found for this nsSNP.⁸ This makes it less likely that it will have an independent effect by itself. Some of the previous reports have obtained evidence of an increased susceptibility due to the concurrent presence of the rare alleles of these two nsSNPs.^{8, 13} We did not find any evidence of this, but due to the rarity of the presence of the two rare alleles in the same subject (expected frequency of about 1%) it will be necessary to use very large sample sizes to address this point adequately.

The most interesting aspect of our study was the suggestion of a particular subgroup of patients where the effect of FRZB seems more likely: women who were recruited because of THR by primary OA and had referred clinical complaints compatible with OA in other joint regions. The strength of the association increased with the number of additional joint regions affected. This phenotype is interesting because it can be regarded as compatible with that found in previous studies and provides a way to conciliate evidence that previously seemed contradictory. In effect, FRZB association with OA has been restricted to women, as in our results, in the only study where sex has been analysed.⁸ Also, only hip OA, apart from polyarticular forms of OA, has been found to be associated with nsSNPs in FRZB. We have expanded the range of OA forms studied to TKR and HOA, but they did not show evidence of association. Finally, there was also a study showing association to polyarticular OA but not to hip OA.¹⁵ We interpret our results as a possible bridge between the association with hip OA and

with polyarticular OA. Against this interpretation, it can be argued that there was not any mention of polyarticular involvement in the reports showing association with hip OA^{8 13 14} or of a particular involvement of the hip joint in the study reporting association with polyarticular OA.¹⁵ Also, a limitation in the comparison of the different studies is that patients and controls have been selected according to different criteria. This is a universal problem in OA genetic epidemiology derived from the variety of clinical presentations of OA, lack of knowledge of the relation between the different forms and differences in emphasis on the different disease features.^{2 3} It is certain that each of the approaches has limitations but they have also advantages over their alternatives. In any case, the association we have found, though related to previous findings, has new aspects and should be considered in this regard as a new hypothesis requiring further confirmation.

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