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Brief report

The *CALM1* core promoter polymorphism is not associated with hip osteoarthritis in a United Kingdom Caucasian population

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Summary

Objective: A convincing genetic association with hip osteoarthritis (OA) of a functional single nucleotide polymorphism (SNP) in the core promoter of the calmodulin 1 gene *CALM1* was recently reported in a Japanese population. The T-allele of the SNP encoded OA susceptibility and this was mediated by a reduced expression of *CALM1*. Our objective was to assess whether the SNP was also associated with hip OA in UK Caucasians.

Methods: The SNP was genotyped in 920 cases that had undergone elective joint replacement of the hip due to end-stage primary OA and in 752 age-matched controls.

Results: Our study had greater than 97% power to observe an effect comparable to that seen in the Japanese study. However, there was no significant difference ($P \leq 0.05$) in genotype or allele frequencies between our cases and our controls. There was also no significant difference when the cases were stratified by sex.

Conclusion: Our data on a cohort of 1672 individuals implies that the *CALM1* core promoter polymorphism is not a risk factor for OA etiology in Caucasians. Our study does not call in to question the veracity of the Japanese report. Instead it highlights the heterogenous nature of OA genetic susceptibility.
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Key words: Osteoarthritis, *CALM1*, Genetic susceptibility, SNP.

Introduction

Through a genome-wide association study, a group recently reported an association of a functional −16C to T transition single nucleotide polymorphism (SNP) rs12885713 within the core promoter region of the calmodulin 1 gene *CALM1* with hip osteoarthritis (OA) in a Japanese population[1]. Calmodulin is an intracellular protein that binds to Ca^{2+} and which interacts with a number of cellular proteins[2,3]. The increased expression of *CALM1* in OA joint cartilage was demonstrated by the Japanese group, as was the ability of calmodulin to up-regulate the expression of *COL2A1* and *AGC1*, the genes that encode the principal cartilage proteins type II collagen and aggrecan, respectively. Calmodulin therefore appears to be an important chondrogenic factor. Ca^{2+}-calmodulin signalling also influences the chondrocyte response to mechanical load, an essential facet of normal articular function.

Possessing two copies (i.e., a recessive model) of the T-allele of SNP rs12885713 was a particular risk factor in the Japanese, with an odds ratio (OR) of 2.56 and *P* value of 0.00036. Functional studies demonstrated that the associated T-allele decreased *CALM1* transcription. The increased OA risk therefore appeared to be acting by reducing the amount of calmodulin synthesised which then resulted in a decreased expression of *COL2A1* and *AGC1*.

Our objective was to assess whether SNP rs12885713 was also associated with OA susceptibility in a Caucasian population collected in the United Kingdom.

Patients and methods

CASE–CONTROL COHORT

The cases ($n = 920$; 547 females and 373 males) were ascertained through the Nuffield Orthopaedic Centre in Oxford using the criteria of signs and symptoms of hip OA sufficiently severe to require hip replacement surgery. All had pain with rest and night symptoms of more than 6 months duration. The radiological stage of the disease was a Kellgren and Lawrence (KL) grade of 2 or more in all cases with over 90% being grade 3 or 4. Inflammatory arthritis (rheumatoid, polyarthritic or autoimmune disease) was excluded, as was post-traumatic or post-septic arthritis. No cases suggestive of a skeletal dysplasia or developmental dysplasia were included. The average age of the cases at hip replacement surgery was 64 years with an age range of 56–85 years. The controls comprised 752 individuals (393 females and 359 males) with no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness or restriction of movement). The average age of the controls at

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recruitment was 69 years with an age range of 55–89 years. Due to ethical and financial constraints the hip joints of the controls were not subjected to radiographic analysis. All cases and all controls were UK Caucasians.

GENOTYPING SNP rs12885713

The SNP was amplified using forward primer 5′GGCGGGACCAGGCGGGCAAG3′ and reverse primer 5′CGTCCACGACACTCCCAGCACCACTGCCGGAGCGC GAGTC*G*GC3′. The italic nucleotide in the reverse primer is a G as opposed to the A that exists in the *CALM1* sequence. This forced change creates a *Bsa*HI restriction enzyme site in the C-allele of the SNP which is absent in the T-allele. The polymerase chain reactions (PCRs) were supplemented with 5% dimethyl sulfoxide (DMSO). The 146 bp PCR product was then digested with *Bsa*HI (New England Biolabs, Hitchin, UK). A C-allele generates two fragments, of 101 bp and 45 bp, whilst a T-allele remains uncut. Digestion products were electrophoresed through 3% agarose and scored following ethidium bromide staining. A second PCR-restriction enzyme genotyping assay was designed to confirm the accuracy of the original assay. The SNP was amplified using forward primer 5′GCGGGAGGCCTGGCGAACCC3′ and reverse primer 5′GGGTACCTCCGATGCCGCTG3′. Neither of these two primers overlapped with the two primers used in the *Bsa*HI assay. The 273 bp product was then digested with *Hpy*CH4V. A C-allele remains uncut whereas a T-allele generates two fragments, of 146 bp and 127 bp.

STATISTICAL ANALYSIS AND STATISTICAL POWER

Genotype and allele distributions in cases and controls were compared using standard chi-square analysis-of-contingency tables. ORs were calculated with 95% confidence intervals (95% CIs). For stratification analysis, female cases were compared to female controls, and male cases were compared to male controls.

To conduct power calculations we used Quanto version 0.5 (http://hydra.usc.edu/gxe/)[4,5] with the following options: (1) 920 cases and 752 controls when using both males and females, 547 female cases and 393 female controls, or 373 male cases and 359 male controls; (2) a recessive model as used by the Japanese group[1] or a dominant model; (3) a population risk of OA of 5%; (4) a T-allele frequency of 57.9%, the frequency for our 752 controls; and (5) a significance level of 0.05.

Results

POWER OF STUDY

Using the Quanto program we determined that our study had greater than 97% power under both dominant and recessive models to detect an OR of 2.40, the smallest OR observed for SNP rs12885713 in the Japanese study[1]. This power was applied for the entire study sample and following stratification by sex.

ASSOCIATION ANALYSIS

Table I lists the genotype and allele frequencies of SNP rs12885713 in our cases and in our controls. The data have also been stratified by sex. We compared the genotype frequencies between the cases and the controls, and the allele frequencies between the cases and the controls. None of the genotype or allele frequencies differed significantly ($P \leq 0.05$). In the Japanese study[1] possessing two copies of the T-allele (genotype [TT], i.e., a recessive model) was a particular risk factor. When we conducted this analysis, there was no significant difference between our cases and our controls.

CONFIRMING THE ACCURACY OF THE GENOTYPING OF SNP rs12885713

The allelic frequencies for SNP rs12885713 differed dramatically between our study and the Japanese study. For example, in our controls ($n = 752$) the T-allele had a frequency of 57.9% whereas in the Japanese controls ($n = 375$) the T-allele had a frequency of 26.4%. This represented a highly significant difference ($P < 0.0001$). This large difference in allele frequency for SNP rs12885713 is noted in the genome databases. In Ensembl (http://www.ensembl.org/) the frequency of the T-allele in 78 unrelated centre d'etude du polymorphisme humain (CEPH) individuals of European ancestry is 83% whereas the frequency in 100 unrelated Japanese is 32%. Such ethnic differences in allele frequency for polymorphic markers are not unusual[6]. However, to be certain that our genotyping was accurate we designed a second PCR-restriction enzyme genotyping assay that used separate primers and a separate restriction enzyme (*Hpy*CH4V) to that used in our original genotyping assay. This second assay was conducted on 90 control individuals. Exactly the same genotypes were obtained for the two assays (data not shown).

Table I
Association of SNP rs12885713 between OA cases and controls from the UK

Group		Genotype			*P* value	Genotype − recessive model		*P* value	Allele		*P* value
		TT	TC	CC		TT	TC + CC		T	C	
All cases ($n = 920$)	Count	296	478	146	0.84	296	624	0.90	1070	770	0.92
	Frequency (%)	32.2	52.0	15.9		32.2	67.9		58.2	41.8	
All controls ($n = 752$)	Count	245	381	126		245	507		871	633	
	Frequency (%)	32.6	50.7	16.8		32.6	67.5		57.9	42.1	
Female cases ($n = 547$)	Count	187	274	86	0.75	187	360	0.97	648	446	0.67
	Frequency (%)	34.2	50.1	15.7		34.2	65.8		59.2	40.8	
Female controls ($n = 393$)	Count	133	191	69		133	260		457	329	
	Frequency (%)	33.8	48.6	17.6		33.8	66.2		58.1	41.9	
Male cases ($n = 373$)	Count	109	204	60	0.84	109	264	0.62	422	324	0.71
	Frequency (%)	29.2	54.7	16.1		29.2	70.8		56.6	43.4	
Male controls ($n = 359$)	Count	112	190	57		112	247		414	304	
	Frequency (%)	31.2	52.9	15.9		31.2	68.8		57.7	42.3	

INVESTIGATING A COMBINATORIAL EFFECT BETWEEN THE *CALM1* SNP RS12885713 AND THE *ASPN* D-REPEAT POLYMORPHISM

The Japanese group that reported the *CALM1* association have also reported an association of large-joint OA to an aspartic acid repeat polymorphism within the *ASPN* gene on chromosome 9q22.31[7]. *ASPN* codes for asporin, which is an extracellular matrix (ECM) macromolecule belonging to the small leucine-rich proteoglycan (SLRP) family[8,9]. The aspartic acid repeat polymorphism was titled the D-repeat and had 10 alleles encoding 10–19 D residues. Allele D14 was associated with hip OA and with knee OA in Japanese. This association appeared to be mediated by a particularly potent inhibitory effect of allele D14 on the anabolic cytokine transforming growth factor-β (TGF-β), which resulted in a reduced expression of the type II collagen gene *COL2A1* and of the aggrecan gene *AGC1*. The associated alleles at *CALM1* and *ASPN* are therefore acting in the same manner, namely reducing the expression of *COL2A1* and *AGC1*. The Japanese investigators had therefore assessed whether *CALM1* and *ASPN* were working together in a combinatorial manner to increase OA risk[1]. The genotype that was [TT] for the *CALM1* SNP rs12885713 and homozygous or heterozygous with respect to the *ASPN* D14 allele proved to be a particularly strong OA risk factor, but of low statistical certainty, with an OR of 13.16 (95% CI 1.66–104.06).

We have previously genotyped the *ASPN* D-repeat in the same hip cohort and in the same controls that we have genotyped here for the *CALM1* SNP[10]. The D-repeat was not associated in our hip cases. When we carried out the combinatorial analysis between the *CALM1* SNP rs12885713 genotype [TT] and the *ASPN* allele D14 we did not detect any evidence of a genotypic interaction (Table II). There was also no evidence of an interaction between the *CALM1* [CT] genotype and *ASPN* allele D14.

Table II
Investigating a combinatorial effect between the CALM1 *SNP rs12885713 and allele D14 of the D-repeat of ASPN*

		All subjects*		Females only†		Males only§	
		OR (95% CI), *P* value		OR (95% CI), *P* value		OR (95% CI), *P* value	
		ASPN					
		D14	Other alleles	D14	Other alleles	D14	Other alleles
CALM1	TT	1.22 (0.76–1.93), 0.43	1.03 (0.72–1.47), 0.98	0.99 (0.51–1.91), 1.0	1.11 (0.69–1.78), 0.65	1.56 (0.78–3.11), 0.20	0.86 (0.48–1.54), 0.68
	CT	1.31 (0.87–1.97), 0.20	1.08 (0.77–1.50), 0.68	1.25 (0.71–2.19), 0.42	1.13 (0.72–1.76), 0.59	1.42 (0.76–2.67), 0.30	1.04 (0.62–1.76), 0.90
	CC	1.04 (0.58–1.88), 0.89	1.00 (reference group)	0.84 (0.37–1.89), 0.71	1.00 (reference group)	1.38 (0.56–3.43), 0.53	1.00 (reference group)

*895 cases and 741 controls.
†533 female cases and 387 female controls.
§362 male cases and 354 male controls.

Discussion

OA is a complex disease with both genetic and non-genetic components[11]. Identifying OA risk alleles is an arduous task complicated by incomplete penetrance, variable expression and a likely high degree of etiological heterogeneity between populations. A number of OA susceptibility loci have been reported and some have been shown to have a role in OA occurrence in more than one study[11]. Our aim was to assess whether a functional SNP within the core promoter of *CALM1* that was strongly associated with hip OA in Japanese had any relevance to hip OA in a Caucasian population. We genotyped a large case–control cohort of 1672 individuals. No association was detected. Our power calculations revealed that we had greater than 97% power to detect an association comparable to that reported in the Japanese. We conclude therefore that the *CALM1* promoter SNP does not influence OA susceptibility in our cohort.

The absence of an association of the *CALM1* functional promoter SNP in our study does not call in to question the Japanese report. Instead, it highlights the complex nature of genetic susceptibility for common multifactorial diseases. Environmental factors or other genetic factors that are more prevalent in the Japanese may influence the extent to which the *CALM1* OA risk is manifest. One interesting difference between Japanese and Caucasians is the shape of the pelvis. There are marked differences in pelvic morphometry between the two populations, with acetabular dysplasia

(AD) demonstrating a significantly greater prevalence in the Japanese[12]. Studies have suggested that AD modestly increases the risk of hip OA[13–15]. In the Japanese *CALM1* study 40% of the patients had AD[1]. However, when the Japanese *CALM1* data were stratified by the presence or absence of AD the association persisted in both groups, implying that the association in Japanese is independent of AD status. Another potentially significant difference between the Japanese cohort and ours was the ascertainment criteria used. Whereas we used the need for joint replacement to ascertain our cases, the Japanese used symptoms and radiological evidence of joint space narrowing and/or the presence of osteophytes. It seems intuitively unlikely, however, that a locus which is a risk factor for the development of symptomatic and radiological OA cannot also be a risk factor for OA that necessitates joint replacement. A further difference between our study and the Japanese was in the proportion of cases that were females. The vast majority of the cases used in the Japanese study were females (94%) whereas in our study the proportion of female cases was 59%. Stratification of our study by sex did not reveal an association to our female cases.

An understanding of the reasons behind ethnic differences in disease risk in response to DNA polymorphism requires a complete knowledge of all potential risk factors and a comprehensive understanding of their interactions. When presented with a compelling association the most that a geneticist can do at present is to test that association in their cohort. Our analysis of the *CALM1* promoter SNP implies that it is not a risk factor for hip OA in the UK population.

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