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Association between *KLOTHO* gene and hand osteoarthritis in a female Caucasian population[1]

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Summary

Objective: Osteoarthritis (OA) is a common complex disease with strong heritable components. In this study, we investigated the association between four putatively functional genetic variants in *KLOTHO* gene, a strong ageing-related gene, and hand OA in a large female Caucasian population.

Methods: Subjects ($n = 1015$, age range 33−74 years) were selected from the TwinsUK Registry. Radiographs of both hands were taken for each individual with standard posteroanterior view. The presence/absence of radiographic OA, osteophyte and joint space narrowing (JSN) was assessed using a standard atlas. Four putatively functional single nucleotide polymorphisms (SNPs) in *KLOTHO* gene were genotyped using allelic discrimination assay. Association was initially estimated using Pearson's χ^2 or Fisher's exact test at allelic and genotypic levels. The direction and magnitude of significant association were further investigated by robust logistic regression with age as a covariate.

Results: We found significant association between SNP G-395A and the presence/absence of radiographic hand OA and osteophyte, but not JSN. Allele G significantly increased the risk for radiographic hand OA and osteophytes with odds ratios (ORs) of 1.44 ($P = 0.008$, 95% confidence interval (CI) 1.09−1.91) and 1.36 ($P = 0.006$, 95% CI 1.09−1.70), respectively. From logistic regression modelling, genotype GG showed more than three-fold increased risk for both radiographic hand OA (OR = 3.10, 95% CI 1.10−8.76) and osteophyte (OR = 3.10, 95% CI 1.10−8.75) when compared to genotype AA. After adjustment for age, ORs for genotype GG further increased to 4.39 ($P = 0.006$, 95% CI 1.51−12.74) for radiographic hand OA and to 4.47 ($P = 0.005$, 95% CI 1.56−12.77) for osteophytes.

Conclusions: Our results suggest that one variant in *KLOTHO* gene is associated with the susceptibility of hand OA and appears to act through osteophyte formation rather than cartilage damage.
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Key words: *KLOTHO* gene, Hand osteoarthritis, Genetic association, Single nucleotide polymorphism.

Introduction

Osteoarthritis (OA) is the most common chronic joint disease characterised by pain, stiffness, limited activity in daily life and eventually disability[1]. OA most frequently affects persons over 45 years and its prevalence is strongly associated with ageing[2]. It affects primarily knees, hips, hands and lower spine. The pathogenesis of OA has been traditionally regarded as a disease mainly caused by breakdown of articular cartilage, but the recent advances suggest it also affects subchondral bone, synovial membrane, muscles, tendons and ligaments. The exact aetiology of OA is largely unknown, but it is typically regarded as a multifactorial disorder with strong genetic components[3-5]. The heritability of OA as estimated from twin studies ranges from 39% to 65% depending

on the joint affected[3,6,7]. In addition, twin studies, linkage and association studies, have generated different results for different sites, which also suggest site-specific genetic influence for OA[8]. Hand OA is the most common type of OA[9].

KLOTHO gene is located on chromosome 13q12. It contains five exons spanning approximately 50 kb of genomic DNA and encodes a type I membrane protein that is related to beta-glucosidases[10]. It has been associated with ageing in several studies[11-17]. The *klotho* homozygous mutated mice ($kl-/-$) manifested a wide range of ageing-related phenotypes, particularly ectopic calcification, osteopenia, osteoporosis and abnormal calcium metabolism[18]. Kawaguchi *et al.*[19] reported that the defect in *klotho* gene expression in homozygous mutated mice ($kl-/-$) causes the independent impairment of both osteoblast and osteoclast differentiation, which leads to low-turnover osteopenia, a representative state for senile osteoporosis in humans. Suzuki *et al.*[20] provided histological evidence that the *klotho* gene deletion influences the spatial distribution of osteocytes and the synthesis of bone matrix proteins in addition to the accelerated ageing of bone cells. Population-based studies also reported significant association between *KLOTHO* variant, G-395A, and bone density in Japanese and Caucasian populations but in opposite directions[15,21].
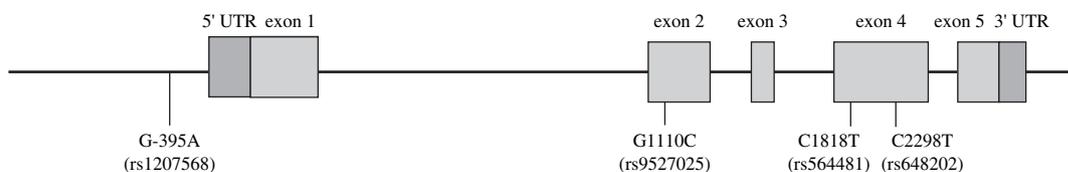
Fig. 1. The positions of four SNPs in *KLOTHO* gene.

Although the *KLOTHO* gene is regarded as an important ageing-related gene, there is no information available on its relationship with OA, a strong ageing-related disease. Therefore, in this study, we attempted to examine the association between *KLOTHO* variants and radiographic hand OA in a female Caucasian population. Four single nucleotide polymorphisms (SNPs) across *KLOTHO* gene have been genotyped and their positions are shown in Fig. 1. One SNP (G-395A) is located in the promoter region and the second SNP (G1110C) is in exon 2. The other two SNPs (C1818T and C2298T) are located within exon 4.

Methods and materials

SUBJECTS

Subjects consisted of 1015 female Caucasian twin individuals recruited through the TwinsUK registry in St Thomas' Hospital, King's College London. Both dizygotic twin (DZ) individuals were included, and only one individual was chosen if they were monozygotic twins (MZ). Demographic data including age, body mass index (BMI) and smoking status were available for each individual. The study was approved by St Thomas' Hospital Research Ethics Committee. All participants were formally informed and consented in writing.

X-RAYS

Radiographs of both hands were taken with a standard posteroanterior view. The distal interphalangeal (DIP), proximal interphalangeal (PIP), metacarpophalangeal (MCP) and first carpometacarpal (CMC) joints of the thumb were assessed for radiographic OA according to Kellgren/Lawrence (K/L) score using a standard atlas[22]. Additional radiographic features including osteophyte and joint space narrowing (JSN) were also assessed on a 4-point scale using another standard atlas[23]. A joint was defined as OA affected if the K/L score is ≥2. Osteophyte or JSN was considered as present if osteophyte or JSN score was ≥1 was considered as the presence of osteophyte or JSN. The X-ray radiographs were independently read by two trained examiners, who were blind to genotype and clinical status of the subjects. A third adjudicator was used in case of disagreement. The X-ray scoring reproducibility within (intra-) and between (inter-) observers was tested in a subgroup of 50 hand radiographs with the reproducibility coefficient

kappa (κ) of approximately 0.68 for all sites[24]. For the purpose of analyses, cases were defined as those individuals with two or more affected joints for each hand OA susceptibility feature. Whereas, individuals with no affected joints were defined as controls.

GENOTYPING

Genotyping was performed using Taqman allelic discrimination assay. ABI Prism 7200 sequence detection platform (Perkin Elmer) was used. Primer and probe concentrations were optimised according to manufacturer's recommendations for each reaction (details available on request). Both FAM-labelled and TET-labelled probes were used. Thermocycle used during Taqman reactions is listed as follows: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s followed by 60°C for 1 min.

STATISTICAL ANALYSIS

Hardy−Weinberg equilibrium test and pairwise Linkage Disequilibrium (LD) were performed for all four SNPs using Haploview[25]. Both allelic and genotypic association tests were performed using Pearson's χ^2 test. Where individual categories had less than five subjects, Fisher's exact test was performed instead. Logistic regression modelling was used to further investigate the direction and magnitude of significance. The strength of association was represented by odds ratio (OR) with 95% confidence interval (CI). Age effect was adjusted for by including age as a covariate in logistic regression models. Robust standard error was estimated from variance between twins to take intra-family effects into account. The association analyses were performed using STATA 9 (Stata Corporation, USA). All statistical tests were two-tailed and the significance level was set at $\alpha = 0.05$.

Results

The characteristics of 1015 subjects are presented in Table I. Cases were on average older (mean ± standard deviation (SD) years = 61.30 ± 6.47 years) than controls (mean ± SD years = 51.21 ± 6.93 years). The prevalence of radiographic hand OA was 24.33% in our samples, which is compatible with previous reports[26]. BMI (kg/m^2) was similar between cases (mean ± SD kg/m^2 = 25.39 ± 3.90 kg/m^2)

Table I
Characteristics of the study subjects

Characteristics	Total ($n = 1015$)	Case ($n = 249$)	Controls ($n = 766$)
Age at X-rays (years ± SD, range)	53.69 ± 8.09 (32.94−74.31)	61.30 ± 6.47 (42.24−74.31)	51.21 ± 6.93 (32.94−72.09)
BMI (kg/m^2 ± SD, range)	24.97 ± 4.36 (16.23−51.68)	25.39 ± 3.90 (16.23−38.21)	24.83 ± 4.50 (16.23−51.68)
% Non-smoking	52.48	52.74	52.37
% of dizygotic twins	84.42	75.50	87.32
% of monozygotic twins	15.58	24.50	12.68

Table II
Details of four genotyped SNPs in KLOTHO gene

SNP	Reference ID	Location	Nucleotide change	Amino acid change	MAF in controls (%)	Hardy−Weinberg equilibrium *P* value
G-395A	rs1207568	Promoter	G/A	−	21.48	0.201
G1110C	rs9527025	Exon 2	G/C	Cys-Ser	15.94	0.163
C1818T	rs564481	Exon 4	C/T	His-His	40.65	0.399
C2298T	rs648202	Exon 4	C/T	Ala-Ala	12.91	0.133

Table III
Allele and genotype frequencies of four SNPs in KLOTHO gene

SNP	Allele	Case (frequency %)	Control (frequency %)	Genotype	Case (frequency %)	Control (frequency %)
G-395A	G	415 (84.01)	1203 (78.53)	GG	172 (69.64)	471 (61.49)
	A	79 (16.00)	329 (21.48)	GA	71 (28.75)	261 (34.07)
				AA	4 (1.62)	34 (4.44)
G1110C	G	408 (82.26)	1276 (84.06)	GG	167 (67.34)	535 (70.49)
	C	88 (17.74)	242 (15.94)	GC	74 (29.84)	206 (27.14)
				CC	7 (2.82)	18 (2.37)
C1818T	C	313 (62.85)	908 (59.35)	CC	102 (40.96)	264 (34.51)
	T	185 (37.15)	622 (40.65)	CT	109 (43.78)	380 (49.67)
				TT	38 (15.26)	121 (15.82)
C2298T	C	420 (85.37)	1329 (87.09)	CC	179 (72.76)	572 (74.97)
	T	72 (14.63)	197 (12.91)	CT	62 (25.20)	185 (24.25)
				TT	5 (2.03)	6 (0.79)

and controls (mean $\pm$ SD kg/m^2 = 24.83 $\pm$ 4.50 kg/m^2). The percentages for zygosity and smoking status were very similar between cases and controls.

The details for genotyped SNPs are listed in Table II. They were all polymorphic in our samples with minor allele frequency (MAF) ranging from 12.91% to 40.65%. All four SNPs were in Hardy−Weinberg equilibrium ($P > 0.05$). Detailed genotype and allele frequencies for the four SNPs are listed in Table III. Three genotypes were present in all four SNPs with the frequency of rare homozygote ranging from 0.79% to 15.82%.

Pairwise LD values (r^2) are shown in Table IV and the pattern is also illustrated in Fig. 2. As r^2 only ranges from 0.02 to 0.13 with low LD between SNPs, haplotype analysis was not performed.

Only one SNP, G-395A, showed significant genotypic and allelic association with the presence/absence of radiographic hand OA and osteophyte, but not with JSN (Table V). Table VI shows the results from logistic regression modelling. The significance was reconfirmed using logistic regression as a whole for radiographic hand OA and osteophyte ($P = 0.037$ and 0.028, respectively), but not JSN ($P = 0.3135$) in the univariate model. For multivariate models as a whole, P values were highly significant ($<10^{-3}$) for all three hand OA susceptibility features. This was mainly due to the strong association between age and hand OA, which was also confirmed by highly significant P values for age as a covariate ($P < 10^{-3}$) within sub-models. When compared with the protective homozygous genotype AA and heterozygote GA, homozygous genotype GG showed a significantly increased risk for radiographic hand OA and osteophytes in univariate models with ORs ranging from 1.34 (95% CI 0.96−1.89) to 3.10 (95% CI 1.10−8.76). After adjusting for age in multivariate models, all ORs further increased. When compared to genotype AA, the risk for genotype GG increased to more than four-fold for both radiographic hand OA and

osteophytes (OR = 4.39, $P = 0.006$, 95% CI 1.51−12.74 and OR = 4.47, $P = 0.005$, 95% CI 1.56−12.77, respectively). Also, the ORs for genotype GG against homozygote AA were more than twice the ORs for homozygote GG against heterozygote GA in both univariate and multivariate models.

Discussion

We found significant association between genetic variation in *KLOTHO* gene and hand OA in a large female Caucasian twin sample set after adjusting for the differences in age.

Although the K/L scoring system has long been regarded as the gold standard for OA classification, it also has been criticised[27]. Therefore, the presence/absence of additional individual features, namely osteophytes and JSN, were investigated rather than using only radiographic hand OA that is purely defined by the K/L score. Although these features are not completely independent, they provide additional information about the different aspects of hand OA pathogenesis.

In our study, SNP G-395A located in the core promoter region of *KLOTHO* gene showed significant allelic and genotypic association with the presence of radiographic hand OA and osteophytes, but not with JSN. The allele

Table IV
Pair-wise linkage disequilibrium (r^2) between four SNPs

SNPs	G-395A	G1110C	C1818T	C2298T
G-395A	......			
G1110C	0.01	......		
C1818T	0.08	0.13	......	
C2298T	0.03	0.03	0.10	......

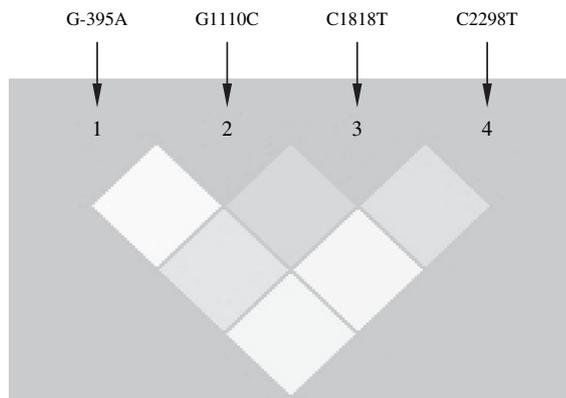G-395A   G1110C   C1818T   C2298T

1   2   3   4

Fig. 2. The illustration of pair-wise LD (r^2) between four SNPs. In this grey-scale representation, the shades of grey between white ($r^2 = 0$) and black ($r^2 = 1$) indicate linkage disequilibrium increments.

Table V
Allelic and genotypic association of G-395A with the presence/absence of hand OA susceptibility features

G-395A allele/genotype	OR (95% CI) for G allele	χ^2	P value
Presence of radiographic hand OA			
G	1.44 (1.09–1.91)	6.98	0.008
A			
GG		7.53	0.021
GA			
AA			
Presence of osteophytes			
G	1.36 (1.09–1.70)	7.61	0.006
A			
GG		8.31	0.015
GA			
AA			
Presence of JSN			
G	1.30 (0.95–1.81)	2.73	0.098
A			
GG		2.60	0.326
GA			
AA			

and genotype frequencies were consistent with previous reports on a Caucasian female population[17,21]. We also demonstrated that the common G allele of G-395A was associated with an increased risk of hand OA. The fact that the significant association was found with radiographic hand OA and osteophytes but not with JSN suggests that the involvement of SNP G-395A in the pathogenesis of hand OA may occur through bone metabolism rather than cartilage damage. Also, as G-395A is located in the promoter region and reported to influence the affinity of binding transcription factors[21], the *KLOTHO* gene might contribute to the susceptibility of hand OA through the regulation of promoter and subsequent expression levels. It is supported by evidence that the abnormal *klotho* expression levels in homozygous mutated mice ($kl-/-$) lead to different ageing-related bone phenotypes, such as differentiation of osteoblasts and osteoclasts, spatial distribution of osteocytes and synthesis of bone matrix proteins[19,20]. But as Kawano and colleagues failed to find the significant difference between allele G and A of G-395A on *KLOTHO* expression levels in human cell culture[21], the potential involvement of SNP G-395A in *KLOTHO* gene function remains speculative. However, the discrepancy might be due to the existence of other important regulatory site(s)

apart from G-395A for the expression of *KLOTHO* and its function[21]. Moreover, G-395A could also act as a surrogate for other functional variant(s) nearby, with which G-395A has strong LD. Further functional studies would be highly desirable to understand the exact mechanism of *KLOTHO* in the pathogenesis of hand OA.

Although our study shows significant association between *KLOTHO* gene and OA, there are several potential limitations. First, all significant association studies require replications in at least one independent sample[28]. Second, although the power of our sample size was above 82% (at significance level of 0.05%) for the common risk allele of G-395A for all three hand OA susceptibility features (data not shown), our sample size might be insufficient to detect real meaningful results for rarer alleles. Therefore, the non-significant results from rarer alleles in our study could not rule out the possibility of real association with hand OA due to limited power. Third, only four SNPs have been genotyped to detect the association for a gene with the size of about 50 kb. The gene coverage

Table VI
Direction and magnitude of genetic association between G-395A and the presence/absence of hand OA susceptibility features

G-395A genotype	Univariate model				Multivariate model with age			
	P*	OR (95% CI)	Robust standard error	P**	P*	OR (95% CI)	Robust standard error	P**
Radiographic OA								
GG vs GA	0.037	1.34 (0.96–1.89)	0.23	0.090	<10^{-3}	1.48 (0.99–2.22)	0.30	0.053
GG vs AA		3.10 (1.10–8.76)	1.64	0.032		4.39 (1.51–12.74)	2.39	0.006
Age						1.22 (1.19–1.26)	0.02	<10^{-3}
Osteophytes								
GG vs GA	0.028	1.40 (0.99–1.97)	0.25	0.058	<10^{-3}	1.58 (1.05–2.37)	0.33	0.029
GG vs AA		3.10 (1.10–8.75)	1.64	0.032		4.47 (1.56–12.77)	2.39	0.005
Age						1.22 (1.18–1.25)	0.02	<10^{-3}
JSN								
GG vs GA	0.314	1.34 (0.82–2.19)	0.33	0.241	<10^{-3}	0.54 (0.15–1.97)	0.36	0.332
GG vs AA		2.24 (0.52–9.59)	1.66	0.279		2.45 (0.73–8.28)	1.52	0.149
Age						1.19 (1.15–1.23)	0.02	<10^{-3}

P* = P value generated from logistic regression as a whole.   P** = P value generated from sub-models by comparison between genotype groups.

might be inadequate to detect all common causative variants as well as the rare ones. Fourth, significant association could also be caused by strong LD between the investigated variant and real causative variant(s) nearby. Fifth, there is a chance of type I error since *P* values were not adjusted for multiple testing. However, as this is an association study with *a priori* hypothesis, the adjustment for non-independent tests would have been over-conservative. Sixth, the results are specific to female Caucasian populations. The effects in males are unknown. Seventh, as our study was conducted in a twin population, the results may not be generalisable. But a previous study conducted by our group found similar rates of OA and comparable lifestyle characteristics between our twin population and population-based singleton samples[29]. Therefore, our results can be generalised to the general population. Finally, the ages between cases and controls were not perfectly matched, but we have adjusted for this in the analyses. Moreover, any possible misclassification of controls in a case−control study would only reduce the significance of the findings.

In summary, the present study shows the association between a genetic variant in the promoter region of *KLOTHO* gene and hand OA in a female Caucasian population for the first time. The results suggest that *KLOTHO* gene plays a role in pathogenesis of hand OA through bone remodelling rather than cartilage damage.

Acknowledgements

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