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Identification of *DIO2* as a new susceptibility locus for symptomatic osteoarthritis

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Osteoarthritis [MIM 165720] is a common late-onset articular joint disease for which no pharmaceutical intervention is available to attenuate the cartilage degeneration. To identify a new osteoarthritis susceptibility locus, a genome-wide linkage scan and combined linkage association analysis were applied to 179 affected siblings and four trios with generalized osteoarthritis (The GARP study). We tested, for confirmation by association, 1478 subjects who required joint replacement and 734 controls in a UK population. Additional replication was tested in 1582 population-based females from the Rotterdam study that contained 94 cases with defined hip osteoarthritis and in 267 Japanese females with symptomatic hip osteoarthritis and 465 controls. Suggested evidence for linkage in the GARP study was observed on chromosome 14q32.11 (log of odds = 3.03, $P = 1.9 \times 10^{-4}$). Genotyping tagging single-nucleotide polymorphisms covering three important candidate genes revealed a common coding variant (rs225014; Thr92Ala) in the iodothyronine-deiodinase enzyme type 2 (D2) gene (*DIO2* [MIM 601413]) which significantly explained the linkage signal ($P = 0.006$). Confirmation and replication by association in the additional osteoarthritis studies indicated a common *DIO2* haplotype, exclusively containing the minor allele of rs225014 and common allele of rs12885300, with a combined recessive odds ratio of 1.79, 95% confidence interval (CI) 1.37–2.34 with $P = 2.02 \times 10^{-5}$ in female cases with advanced/symptomatic hip osteoarthritis. The gene product of this DIO2 converts intracellular pro-hormone-3,3′,5,5′-tetraiodothyronine (T4) into the active thyroid hormone 3,3′,5-triiodothyronine (T3) thereby regulating intracellular levels of active T3 in target tissues such as the growth plate. Our results indicate a new susceptibility gene (*DIO2*) conferring risk to osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is the most common age-related disabling joint disease, characterized by degeneration of articular joint cartilage. The existing drugs mainly intervene in symptoms such as pain and joint function but do not reverse the disease process itself. In the Western world, OA ranks fourth in

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Table 1. Characteristics of GARP sibling pairs and cases and controls of the UK, Rotterdam and Japanese study

	GARP study[a]		UK study[b]		Rotterdam study[b]	Japanese study[b]	
	GARP	Controls	Replacements	Controls		Cases	Controls
Number	370	714	1478	734	1582	267	465
Age (range)[c]	60 (43–80)	60 (55–65)	65 (56–85)	69 (55–89)	67 (55–89)	60 (11.2)	62 (14.2)
BMI (SD)	27.0 (4.6)	26.3 (3.7)	—	—	26.7 (3.9)	22.8 (3.3)	23.1 (3.8)
Women (%)	301 (81)	430 (60)	835 (57)	377 (51)	1582 (100)	267 (100)	465 (100)
Hand (%)	266 (72)	—	—	—	—	—	—
Spine (%)	294 (79)	—	—	—	—	—	—
Hip (%)	91 (25)	—	1115 (75)	—	94 (6)	267 (100)	—
Knee (%)	128 (34)	—	363 (25)	—	—	—	—

[a]Overall, the GARP study consists of 187 pairs and four trios. Characteristics are shown on 179 sibling pairs and four trios that were included in the genome-wide linkage analysis. In the association analyses, genotypes and haplotypes (posterior probability ≥ 0.5) were available on 714 random controls and 360 GARP subjects (of these 113 were IBD2 for the *DIO2* locus).
[b]Genotypes and haplotypes (posterior probability ≥ 0.5) were available in UK study on 712 controls and 1458 cases, in Rotterdam study on 1306 random subjects and 84 hip cases and in Japanese study on 267 hip cases and 465 controls.
[c]In Japanese study standard deviation is provided behind brackets.

Table 2. Markers and chromosomal regions yielding an LOD-score >1.0 in genome-wide NPL analyses of affected sibling pairs of the GARP study

Chromosome	Markers			Linkage peak		
	Interval (cM)	Position (cM)	Informativity[a]	Interval (cM)[b]	Position (cM)	Maximum LOD[c]
6	D6S1574	14	0.56	0–43	0	1.13
10	D10S196	69	0.33	48–84	69	1.36
13	D13S175	1	0.38	0–12	0	2.23
14	D14S74; D14S280	76; 92	0.39; 0.49	32–117	83	1.32

[a]Informativity at the position of the marker.
[b]One LOD-drop interval.
[c]Maximum LOD score multipoint analysis plotted on a 1 cM grid.

health impact among women and eighth in men (1). OA is also regarded as a complex genetic disease the etiology of which is not completely understood. Elucidation of common pathways that are involved in the onset and progression of the disease may assist in the development of new drug targets and a better management of this disabling condition in the future.

In the search for susceptibility loci for OA, linkage and association studies mainly focused on OA at a single joint location with definitions based on either radiographic or symptomatic criteria alone. These efforts have yielded several consistent susceptibility loci and some responsible genes which highlights the complexity of OA (2–5). A number of loci identified in Japanese and Chinese patients were not as relevant for Caucasian patients, indicating additional ethnic differences regarding the OA phenotype between Japanese and Caucasian individuals (6–9).

In the present study, we focus on the identification of new susceptibility loci in 179 affected siblings and four trios with symptomatic OA at multiple joint sites (the GARP study) by performing a genome-wide linkage scan and combined linkage association. We tested for confirmation by association in independent UK, Dutch and Japanese OA studies.

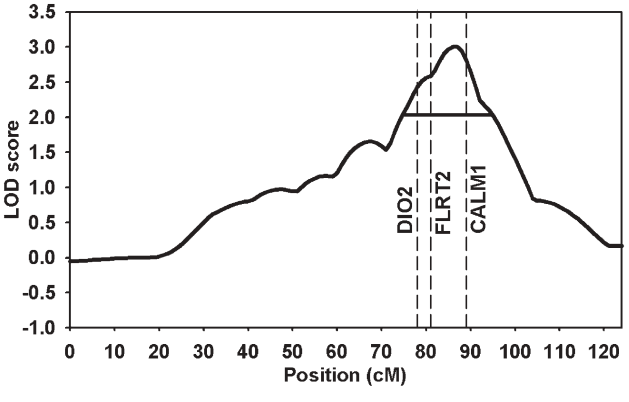

Figure 1. Suggestive evidence for linkage was observed on chromosome 14q32.11 using 179 Caucasian sibling pairs and four trios from the GARP study. Horizontal line represents one LOD-drop interval [75–95 cM; mean informativity = 0.50 (range, 0.46–0.56)]. Three vertical lines represent the genes *DIO2* (78 cM; informativity = 0.51), *FLRT2* (82 cM; informativity = 0.54) and *CALM1* (89 cM; informativity = 0.47).

RESULTS

Genome-wide linkage scan of the GARP study

Initial genome-wide non-parametric linkage (NPL) analysis in the GARP study (Table 1) provided several suggestive linkage signals on chromosomes 6, 10, 13 and 14 (Table 2). Typing 14 additional markers in these four areas reduced the evidence for linkage on chromosomes 6, 10 and 13. In contrast, the log of odds (LOD) score on chromosome 14q32.11 increased from 1.32 ($P = 0.007$) to 3.03 ($P = 1.9 \times 10^{-4}$), corresponding to a global P for chromosome 14 of 0.0013 and a genome-wide P of 0.0299 (Bonferroni corrected for 23 chromosomes). The one LOD-drop interval of this signal encompasses 22 cM

Table 3. Allele frequencies of *DIO2* SNPs in GARP sibling pairs stratified for IBD status

SNP	Allele	IBD = 0 MAF[a]	IBD = 1 MAF[a]	IBD = 2 MAF[a]	P[b]
rs12885300	C > T	0.34 (48/142)	0.38 (134/354)	0.33 (77/234)	0.04
rs2267872	G > A	0.11 (15/142)	0.11 (37/344)	0.07 (16/232)	0.30
rs225011	T > C	0.44 (62/142)	0.40 (137/343)	0.48 (110/230)	0.14
rs225014	T > C	0.35 (49/142)	0.32 (111/348)	0.44 (102/232)	0.006
rs10136454	C > T	0.007 (1/143)	0.014 (5/358)	0.029 (7/240)	0.60

[a]Minor allele frequency (MAF) stratified for IBD status (sum of minor alleles/sum of total alleles).
[b]P value observed using combined linkage and association with the program LAMP (15).

(75–95 cM) containing the markers D14S74, D14S1037, D14S1044 and D14S280 (Fig. 1). Among the pairs that showed a positive LOD score at this locus we could find no significant correlation between the LOD score per family and any specific combination of affected joint sites. Notably, the location of the linkage peak coincided with the Calmoduline gene *CALM1* [MIM 114180], previously associated with hip OA in the Japanese population (5). A search of public genome resources revealed two other attractive candidate genes within the linkage area: fibronectin–leucine-rich transmembrane protein 2 gene (*FLRT2* [MIM 604807]), encoding a small molecule found in the extracellular matrix of cartilage (10), and *DIO2*, encoding for Iodothyronine-deiodinase enzyme type 2 (D2), a selenoprotein that converts intracellular inactive thyroid hormone (T4) to active thyroid hormone (T3) (Fig. 1). D2 is an important provider of local bioactive T3 in target tissues such as growth plates (11–14).

Combined linkage association in the GARP study

To examine whether genetic variation in these three genes explains the observed linkage signal, tagging single-nucleotide polymorphisms (SNPs) covering the haplotype blocks in which these genes reside were genotyped in the GARP cohort. Joint modeling of linkage and association by using linkage and association modeling in pedigrees (LAMP) revealed no evidence for association with the putative disease locus of individual SNPs in *CALM1* (including the functional SNP rs12885713) or *FLRT2* (Supplementary material, Table S1). However, a significant predisposing association with the C allele of *DIO2* SNP rs225014 ($P = 0.006$), a protective association with the T allele of *DIO2* rs12885300 ($P = 0.04$) and a putative disease locus were found (Table 3). To confirm this by a more robust method than LAMP, allele frequencies in sibling pairs sharing two alleles identical by descent (IBD) at the *DIO2* locus (indicating those subjects that contribute to the linkage) were compared with allele frequencies of those subjects that did not contribute to the linkage or to random controls. With this approach, the frequency of the C allele of rs225014 was again significantly increased among subjects that contributed to the linkage when compared with those subjects that did not contribute to the linkage ($P = 0.0034$) or to random controls ($P = 0.025$). The T allele of *DIO2* SNP rs12885300 did not show significant association (data not shown). The complete linkage disequilibrium (LD) test of LAMP between rs225014 and the disease locus was rejected ($P = 0.002$), indicating that the SNPs were able to explain only part of the linkage signal. In general, it is known that combined linkage association approaches have limited power for complex diseases when multiple (rare) genetic variants are expected to be involved (15). We, therefore, expect that for the GARP subjects contributing to the linkage additional relevant genetic variation resides in the region of linkage.

Subsequently, we investigated the haplotypic combined linkage association effects of the *DIO2* SNPs rs12885300 (C>T) and rs225014 (T > C) in GARP. Three common haplotypes with frequencies >0.05 were revealed (Supplementary material, Table S2). The common haplotype C-C (frequency 0.34) exclusively carried the minor allele of *DIO2* SNP rs225014 and the common allele of SNP rs12885300 (Supplementary material, Table S2). This haplotype showed a significant combined linkage association among GARP sibling pairs sharing two alleles IBD when compared with allele frequencies of random controls ($P = 0.016$) or when compared with allele frequencies of GARP subjects that did not contribute to the linkage ($P = 0.0027$). When testing the effects of genotype differences between GARP sibling pairs sharing two alleles IBD and controls, the dominant model showed most optimal association with an OR 2.03, 95% CI 1.21–3.43 and P-value of 0.008. The GARP study consists mainly of females (81%) which hamper robust assessment of gender-specific effects. Stratified analysis, however, indicated that both females (1.99, 95% CI 1.13–3.52, $P = 0.018$) and males (2.39, 95% CI 0.99–5.80, $P = 0.054$) contributed to this association. As the analysis of the haplotype revealed a slightly larger effect than the allele, we used the C-C haplotype for rs225014 and rs12885300 in subsequent analyses. Possibly this haplotype carries functional variation in LD with the rs225014 affecting OA susceptibility. Because of the small difference between the allelic and haplotypic effect, *DIO2* rs225014 may still itself be functionally relevant.

Confirmation and replication in independent UK, Dutch and Japanese OA studies

For confirmation of our findings, patient populations with the same generalized OA phenotype as present in the GARP study were not readily available. We, therefore, attempted to confirm the observed association of the *DIO2* C-C haplotype as potential OA susceptibility gene in a UK population that consists of 363 and 1115 subjects who required replacement of knee or hip, respectively, because of OA signs and symptoms and 734 controls (Table 1) (16). As in the GARP study, three common haplotypes with frequencies >0.05 were revealed (Supplementary material, Table S2). For haplotype C-C, a modest predisposing genotypic association was revealed for cases when compared with controls (Table 4; $P = 0.038$). Stratified analysis established that this effect was mainly driven by female subjects with hip replacements (Table 4; $P = 0.002$ in genotypic frequency and $P = 0.037$ in allele frequency). In the UK sample, this haplotype revealed the most significant association with the recessive model in females

Table 4. Association of *DIO2* haplotype rs12885300 (C>T) and rs225014 (T>C) SNP rs225014 C-C between subjects with hip and/or knee replacement due to OA and controls from the UK

Group		Copies of C-C			P^a	Haplotype		P^a
		0	1	2		Others	C-C	
All controls	Count	310	337	65		957	467	
	%	43.5	47.3	9.1		67.2	32.8	
All cases	Count	645	631	182	0.038	1921	995	0.385
	%	44.2	43.3	12.5		65.9	34.1	
Female controls	Count	162	172	30		496	232	
	%	44.5	47.3	8.2		68.1	31.9	
Female cases	Count	356	352	119	0.011	1064	590	0.072
	%	43.0	42.6	14.4		64.3	35.7	
Male controls	Count	148	165	35		461	235	
	%	42.5	47.4	10.1		66.2	33.8	
Male cases	Count	289	279	63	0.646	857	405	0.450
	%	45.8	44.2	10.0		67.9	32.1	
All knees	Count	164	160	33	0.728	488	226	0.595
	%	45.9	44.8	9.2		68.3	31.7	
Female knees	Count	87	92	20	0.771	266	132	0.656
	%	43.7	46.2	10.1		66.8	33.2	
Male knees	Count	77	68	13	0.411	222	94	0.206
	%	48.7	43.0	8.2		70.3	29.7	
All hips	Count	481	471	149	0.010	1,433	769	0.187
	%	43.7	42.8	13.5		65.1	34.9	
Female hips	Count	269	260	99	0.002	798	458	0.037
	%	42.8	41.4	15.8		63.5	36.5	
Male hips	Count	212	211	50	0.728	635	311	0.705
	%	44.8	44.6	10.6		67.1	32.9	

[a]*P*-values of χ^2 statistics for specific case group versus the specific control sample.

with hip replacement with an odds ratio of 2.08, 95% CI 1.35–3.21 with nominal two-tailed $P = 0.001$ (Table 5). Furthermore, as shown in Supplementary material, Table S2, a haplotypic frequency deviation was observed for the T-T haplotype. However, this frequency difference was driven solely by the UK female control group, showing unexpected high frequency of the T allele and was therefore disregarded.

Following this confirmation, we tested in two additional independent OA studies whether replication can be found for recessive association of the C-C haplotype to women with advanced symptomatic hip OA as defined in the Materials and methods. First, we tested a set of 1582 females from the Rotterdam population-based study in which we, *a priori*, defined 94 (6%) female cases with advanced/symptomatic hip OA. Using an alternative genotyping method, the recessive association with the C-C haplotype in this case group was replicated when compared with others as control. The strength of the association was obtained using logistic regression analyses adjusted for age and body mass index (BMI) and indicated an OR of 1.89, 95% CI 1.03–3.48, with nominal two-tailed $P = 0.040$ (Table 5).

Secondly, we investigated whether the association could be replicated in a population of different ethnic origins by investigating Japanese females with symptomatic and radiographic confirmed hip OA without signs of acetabula dysplasia ($N = 267$) as case group compared with random Japanese controls ($N = 465$). This study population is described in detail previously by Miyamoto *et al.* (4). As shown in Supplementary material, Table S2, ethnic differences in the allele frequency of the rs12885300 were observed that resulted in a significantly different haplotype distribution. However, the *DIO2* haplotype frequency rs12885300 (C > T)–rs225014 (T > C) C-C appeared stable across the ethnic populations. Again a significant recessive association was observed for the *DIO2* haplotype rs12885300 (C > T)–rs225014 (T > C) C-C with an OR of 1.52, 95% CI 1.01–2.29, with nominal two-tailed P = 0.047 (Table 5).

To assess the overall effect of the DIO2 C-C haplotype in females with advanced/symptomatic hip OA, meta-analyses were performed in the confirmation and replication studies. As depicted in Table 5, genotype differences between cases and controls showed a significant recessive association in the confirmation/replication studies with an odds ratio 1.79, 95% CI 1.37–2.34 ($P = 2.0 \times 10^{-5}$) without any evidence for heterogeneity across the studies ($I^2 = 0\%$, P = 0.569). These data confirm recessive association to symptomatic hip OA in women. Significant heterogeneity and absence of association was observed only when we tested the dominant model (Supplementary material, Table S3). None of the SNPs in the studies revealed a departure from Hardy–Weinberg equilibrium in controls.

LD and haplotype analysis of *DIO2* gene

Using HapMap (17), we evaluated the LD extension of rs225014 and rs12885300 in both Caucasian and Japanese subjects to exclude other possible susceptibility genes or variants present in this region, in LD with these SNPs. As shown in Supplementary material, Figure S1, flanking the *DIO2* gene recombination hotspots defines a ~260 kb interval containing *DIO2* as only known gene. In this interval pair, wise-LD analysis of rs225014 and rs12885300 with all other SNPs displayed D' scores greater than 0.90, however, the LD readily decreases when approaching the recombination hotspots (Supplementary material, Fig. S1). It is, therefore, unlikely that the LD of the haplotype containing the SNPs rs12885300 and rs225014 extends beyond this haplotype block and that the current observed association originated from other susceptibility genes.

DISCUSSION

By performing a genome-wide linkage scan in sibling pairs of the GARP study, we have identified *DIO2*, encoding the D2 enzyme determining the availability of local active thyroid, as a new susceptibility gene for OA. Meta-analyses of three additional independent OA studies of Caucasian and Asian descent confirmed this locus among females with advanced symptomatic hip OA showing a significant recessive association of the *DIO2* haplotype rs12885300 (C > T)–rs225014 (T>C) C-C with an OR of 1.79, 95% CI 1.37–2.34 ($P = 2.02 \times 10^{-5}$) without any evidence for heterogeneity across these studies. Significant associations were also observed for the haplotype frequency and trend test but not for the dominant model (Supplementary material, Table S3).

Heterogeneity for the DIO2 effect, however, was observed between the 'discovery' study GARP and the confirmation/

Table 5. Random effects meta-analysis of confirmation/replication studies UK, Rotterdam and Japanese for the haplotypic association of *DIO2* SNPs rs12885300 (C>T) and rs225014 (T>C) C-C with OA

Summary	Copies of C-C (frequency) Controls			Cases[a]			Recessive Model OR (95% CI)	*P* of OR (*z*-score)	*P* of heterogeneity test (I^2%)
	0	1	2	0	1	2			
Confirmation / replication studies[b]	—	—	—	—	—	—	1.79 (1.37–2.34)	2.02×10^{-5}	0.569 (0%)
UK	162 (0.45)	172 (0.47)	30 (0.08)	269 (0.43)	260 (0.41)	99 (0.16)	2.08 (1.35–3.21)	0.001	
Rotterdam	550 (0.42)	616 (0.47)	140 (0.11)	29 (0.35)	41 (0.49)	14 (0.17)	1.89 (1.03–3.48)	0.040	
Japan	167 (0.36)	238 (0.51)	60 (0.13)	107 (0.40)	111 (0.42)	49 (0.18)	1.52 (1.01–2.29)	0.047	

Heterogeneity was evaluated using the Cochran *Q* test and I^2-values (%).
[a]UK female cases had hip replacements, Rotterdam female cases had severe radiographic hip OA (KL score $\geq$ 3) and Japan female cases had radiographic and clinical signs of OA without dysplasia. Together cases were defined as having advanced/symptomatic hip OA.
[b]Combined OR of confirmation/replication studies UK, Rotterdam and Japan. For the Rotterdam study, age and BMI were added as co-variables in the analyses.

replication studies considering the optimal genetic model (dominant versus recessive), OA subtype (generalized versus hip) and gender specificity. In general, this may reflect differences in ascertainment schemes, heterogeneity in the phenotype, sample sizes, environmental influences or in the genetic background which are likely to account also for the current observed differences. In contrast to the confirmation/replication cohorts, ascertainment of the GARP study was based on symptomatic OA at multiple joint sites and familial background in order to include sibling pairs and to enrich for genetically predisposed subjects. As such, the selection criteria in the GARP study do not allow for the stratification of cases with hip OA only. Stratification by joint site or sex, however, did not reveal evidence that the *DIO2* susceptibility was only limited to female subjects with hip OA (data on hip OA not shown). Data regarding the presence of symptomatic OA at sites other than the hip are not taken into account in the confirmation/replication studies. Especially among the advanced symptomatic female hip OA cases there may be those that have symptomatic OA at other sites, thus becoming comparable to GARP patients. With respect to the optimal association model, it should be noted that the GARP study showed linkage to the chromosome 14 region, which was only partly explained, in the combined linkage association analysis, by the *DIO2* susceptibility allele. Hence, additional genetic variation affecting OA susceptibility in resides in this region which may consist of 'private' rare variants in the DIO2 gene and/or OA susceptibility alleles (common and/or rare) in other positional candidate genes. As such, compound heterozygotes for the *DIO2* haplotype C-C and additional susceptibility alleles in this region among GARP subjects may explain the observed dominant model of association. To find such rare variants warrants sequencing of the heterozygous GARP subjects. Possible reasons for the fact that we were not able to detect such additional susceptibility alleles may be lack of power of the GARP study or insufficient SNP coverage of the positional OA (candidate) genes in the combined linkage and association analyses.

By applying strict inclusion criteria in the GARP study, affected sibling pairs with symptomatic OA in combination with radiology at multiple joint sites, we have aimed to include genetically predisposed subjects with reasonably high penetrance. As previously indicated, the prevalence of this phenotype has not been established (18). Given the fact that symptomatic OA is known to be less frequent than radiographic OA and our experience during GARP recruitment, we consider the GARP phenotype relatively rare. As such, a prevalence of 0.01 in the LAMP analyses was applied. Changing the prevalence from 0.01 to 0.05, however, did not considerably change the LAMP output (data not shown).

Because of the recent advances in genome-wide association (GWA) studies to identify susceptibility loci, methods for significance testing have become a subject of major importance. As pointed out by Skol *et al*. (19), joint analysis of multiple stages of samplings in GWA studies is most efficient despite the requirement of stringent *P*-values of $\leq1\times10^{-7}$, as the initial 300,000–500,000 SNP tests should be taken into account. In comparison, it was indicated that a replication strategy, although less powerful in the GWA, may consider significant *P*-values defined by $\alpha = 0.05$/the number of markers tested in the replication studies (19). These statements are in line with other papers on the subject (19–20) and the consensus appears to be that *P*-values of $\leq10^{-4}$ should be considered significant for candidate gene approaches. Given the design of the current study, the joint analysis of the original linkage results with the results from additional case–control association designs may not be most straight forward, but does not suffer from an initial 500,000 tests. Instead, following the most conservative reasoning for candidate gene approaches, the results shown in Table 5 may be considered significant.

The *DIO2* SNP rs225014 is a non-synonymous coding SNP resulting in the amino acid change Thr92Ala. Residue 92 is the first amino acid of the instability loop in D2 and this loop is the key determinant of D2 turnover rate (14). *In vitro* studies have indicated that in HEK293 cells there is no functional effect of the 92Ala allele *per se* (22,23). However, Canani *et al*. (23) showed *in vivo* a decreased D2 velocity in skeletal muscle and thyroid tissue in subjects homozygous for the 92Ala allele as result of a decreased maximum enzyme velocity. This effect was observed in the absence of differences in D2 mRNA level or in the biochemical protein

properties of the 92Ala allele. It was, therefore, suggested that either a functionally relevant SNP occurs in LD with rs225014 or a direct effect of the 92Ala allele occurs on protein translation or stability.

Functional relevance on enzyme activity was shown *in vitro* by others for *DIO2* SNP rs12885300, which is localized in a short open reading frame a (ORFa) within the 5′-untranslated region of the gene and also known as D2-ORFa-Gly3Ala (24). The ORFa has been shown to reduce the D2 translation efficiency which is abolished by mutating the start codon (25) and weakened by the amino acid modification due to SNP rs12885300 (26). Together, these studies indicate inverse functional effects of the *DIO2* SNP alleles rs12885300 3Ala and rs225014 92Ala on D2 activity. This is consistent with the mutually exclusive appearance of the 3Ala and 92Ala alleles on the *DIO2* gene haplotypes. As shown in Supplementary material, Table S2, the D2-92Ala allele resides exclusively on the predisposing haplotype C-C, whereas the D2-ORFa-3Ala allele resides exclusively on haplotype T-T. In view of the results of the current paper, we hypothesize that decreased D2 enzyme activity resulting in decreased availability of intracellular active T3, creating a state of relative intracellular hypothyroidism and increases OA susceptibility. The exact (opposite) interplay and functional relevance of the *DIO2* SNPs rs225014 and rs12885300 in OA needs to be investigated further in cartilage tissue. Pathophysiologically, D2 as a key regulator of local T3 availability may contribute to OA development in different ways. In the growth plate, the conversion of T4 into T3 by D2 inhibits chondrocyte proliferation but stimulates chondrocyte differentiation and subsequently bone matrix synthesis. This process of endochondral ossification is essential for the formation of the skeleton (27). Lower expression of D2 in the growth plate has been shown to contribute to the pathogenesis of tibial chondrodysplasia in chicken (28). Furthermore, in human it has been recognized that hypothyroidism is associated to early-onset osteoarthropathy involving hypertrophic chondrocytes and sometimes short stature (29,30). In the GARP study, homozygous female carriers of the minor allele of rs225014 were significantly shorter (mean height 162 cm) when compared with other carriers (mean height 166 cm, $P = 0.001$), stressing that skeletal development and growth may be involved in OA susceptibility in this study. Alternatively, altered D2 function may contribute to OA progression. In OA cartilage, chondrocytes, in an attempt to repair damaged matrix, show increased metabolic activity. During progression of OA, chondrocytes undergo phenotypic dedifferentiation to a hypertrophic state expressing similar features as chondrocytes residing in the growth plate (31). As such, chondrocyte hypertrophy debilitates cartilage viability by a switched expression of bone-specific collagens which initiates calcification of the matrix and up-regulation of cartilage-specific proteolytic enzymes (32).

Furthermore, it was recently shown that inflammatory signals up-regulate D2 expression via a dimer combination of RelA (p65) with nuclear factor-κB (NF-κB) (33).This p65/NF-κB dimer has also been shown to activate pro-inflammatory cytokines in chondrocytes mediating cartilage degeneration. Finally, OA is characterized by formation of bony enlargements at the edges of the bone called osteophytes, a process which is characterized by endochondral ossification (34).

Altogether deficiency of D2, as a key regulator of the endochondral ossification process, may predispose to the incidence of OA via its influence on skeletal formation or later in OA cartilage via its influence on viability of chondrocytes and formation of osteophytes. Our findings underscore the importance of local thyroid hormone availability in the etiology of symptomatic OA. We are confident that knowledge of genetic factors and their molecular cascades contributes to a better understanding of the pathogenesis and management of OA and will lead to improved diagnosis, treatment and prevention.

MATERIALS AND METHODS

Subjects

The Genetics osteoARthritis and progression (GARP) study consists of 187 Caucasian sibling pairs and four trios of Dutch origin affected by symptomatic and radiographic OA at multiple sites (18). Radiographic OA was assessed according to the Kellgren–Lawrence (KL) (0–4) method (35), whereas symptomatic OA was defined according to the American College of Rheumatology (ACR) recommendations (36–38). Detailed phenotypic description of the GARP study can be found in Riyazi *et al*. (18). In the association and haplotype analysis, we compared affected sibling pairs from the GARP study carrying 2 alleles IBD to a random sample of unrelated subjects aged 55–65 years of the Rotterdam study as reference group representing the general population. Both studies comprise Caucasian subjects from the western areas of the Netherlands with a mean age of 60.3 years and may represent the same genetic background.

In the confirmation and replication studies, radiographic OA was assessed by the KL (0–4) method (35). Subjects of the UK study were ascertained using the criteria of signs and symptoms of OA sufficiently severe to require joint replacement surgery. All had pain with rest and night symptoms of more than 6 months duration. The radiographic stage of the disease was a 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 replacement surgery was 65 years with an age range of 56–85 years. The controls comprised individuals with no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness or restriction of movement). The average age of the controls at recruitment was 69 years with an age range of 55–89 years. Because of the ethical and financial constraints, the hip joints of the controls were not subjected to radiographic analysis. All cases and controls were UK individuals of white European ethnicity.

The Rotterdam study, which comprises 7983 Caucasian participants, is a prospective, population-based cohort study of the determinants and prognosis of chronic diseases in the elderly (39). The Rotterdam replication sample consists of women aged ≥ 55 years ($N = 1582$) for which radiographs of the hip were scored for the presence of OA according to

the KL grading system (grades 0–4). Symptomatic OA has not been assessed (40). In order to select for comparable female cases as in the UK study (females with sufficiently severe symptomatic hip OA to require hip replacement) and in the absence of symptomatic data, Rotterdam females with hip OA defined by a KL grade of 3 or more were considered. Average age was 67 (55–89) years and BMI was 26.7. Subjects did not show overlap to the Rotterdam control samples mentioned above.

The Japanese case–control subjects were recruited through several medical institutes in Japan as previously described in detail (4). Affected females showed symptoms and radiographic signs of OA in the hip and were compared to random controls. Females with acetabular dysplasia were excluded. Written informed consent was obtained from each subject as approved by the ethical committees of the SNP Research Center at RIKEN and participating clinical institutes.

In the association analyses, cases of the GARP study were defined as having symptomatic OA at multiple joint sites, whereas cases of the confirmation and replication studies were defined as advanced/symptomatic hip OA. Case definitions were assessed prior to association analyses.

Genotype measurements

Short tandem repeat polymorphisms. A complete genome-wide scan containing 403 microsatellite markers with an average spacing of 10 cM was performed in the GARP study. Fourteen additional microsatellite markers for fine mapping on chromosomes 6, 10, 13 and 14 were taken from Human Linkage Set v2.5 MD10 or HD5 (Applied Biosystems) and measured using an ABI Prism DNA Analyzer 3700 (Applied Biosystems). Genotyping was performed using standard conditions and reagents with some exceptions. The amount of polymerase chain reaction (PCR) primer pairs for the markers was reduced up to 5-fold, and duplex PCR reactions were designed if possible to reduce costs, time expense and amount of genomic DNA used. Genotypes were analyzed by using Genemapper versions 2.0 and 3.0 (Applied Biosystems). As quality control, ~8% of the samples were genotyped in duplicate and compared. In addition, 48 additional family members from 36 different sibling pairs were genotyped to improve our ability to detect genotyping errors and estimate allele sharing. Mendelian errors were checked for Mendelian inconsistencies and unlikely recombinants using Merlin (41). These quality checks indicated that markers D6S434 and D9S158 from the Human Linkage Set v2.5 MD10 could not be genotyped reliably in our hands because of unclear base pair differences. Subjects and markers showed an average success rate of 96% (range 77–100%) and 96% (range 83–100%), respectively. Family relationships were verified using the GRR program (42). Eight sibling pairs showed pedigree errors and were removed for further analysis. In seven of these sibling pairs, individuals reported to be full siblings were almost certainly half siblings. The remaining siblings were monozygotic twins. A locally developed SQL database was used to store genotypic data, to compare repeated genotypes and to generate output files for linkage analysis. The location of the markers was taken from an integrated genetic map of David Duffy with interpolated genetic map positions (http://www2.qimr.edu.au/DavidD/). The position is in Decode cM, estimated via locally weighted linear regression (lo(w)ess) from the Build 35.1 (and 34.3) physical map positions and published Decode and Marshfield genetic map positions.

Single-nucleotide polymorphisms. Subjects of the GARP study were genotyped for highly informative tagging SNPs capturing a large fraction of all genetic variations in the *CALM1*, *DIO2* and *FLRT2*. Tagging SNPs were selected from HapMap Public Release #19 applying the efficient multimarker method with $r^2 > 0.8$ and minor allele frequency (MAF) > 0.05 as implemented in the HapMap web browers (http://www.hapmap.org) (43). For the genomic region of the *DIO2* gene, we were able to capture 14 out of 14 (100%) alleles at $r^2 > 0.8$. For the genomic region of the *CALM1* gene, we were able to capture 11 out of 13 (84%) alleles at $r^2 > 0.8$. For the genomic region of the *FLRT2* gene spanning 98 kb, we were able to capture 43 out of 139 (30%) alleles at $r^2 > 0.8$. However, 80% of the genes span 6.8 kb for which we were able to capture 43 out of 45 alleles (95%). Tagging SNPs or their proxies were chosen to fit efficiently in a Sequenom multiplex assay. The following tagging SNPs were genotyped in *CALM1*: rs3814847, rs3814845, rs2300496, rs2300502 and rs5871. For *DIO2*, we measured rs225014, rs2267872, rs225011, rs12885300 and rs10136454. For *FLRT2*, rs2239576, rs2057311, rs17121375, rs17646457 and rs1129671 were genotyped. Multiplex genotyping assays were designed using Assay Designer software (Sequenom, San Diego, CA, USA). Tagging SNPs were genotyped by mass spectrometry (the homogeneous MassARRAY system; Sequenom, San Diego, CA, USA) using standard conditions. PCR reactions were carried out in a final volume of 5 μl and contained standard reagents and 2.5 ng of genomic DNA. Genotypes were assigned by using Genotyper version 3.0 software (Sequenom, San Diego, CA, USA). The functional SNP rs12885713, located in *CALM1*, and the *DIO2* SNPs rs12885300 and rs225014 in the Rotterdam study were genotyped using a Taqman by design assay and an ABI Prism DNA Analyzer 7900 (Applied Biosystems) with standard conditions. SNP genotype success rates were >95% for each (follow-up) study and genotype platform. However, for SNPs rs2267872, rs225011, rs225014 and rs3814847, a success rate of 89% was observed in the GARP study. Genotype distributions of all SNPs were in agreement with the Hardy–Weinberg equilibrium and ~8% of the subjects were genotyped twice and checked.

Statistical analysis

Linkage analysis. NPL analysis was carried out by use of the S_{pair} statistics (44) implemented in the software Merlin (41). LOD scores were plotted on a common 1 cM grid. For X-chromosome analyses, we used MINX (Merlin-In-X). The variance of the NPL test statistic was computed by Monte Carlo simulations of marker genotypes under the H_0 using Merlin. A global *P*-value for chromosome 14 was computed by smoothing the likelihood with respect to a uniform prior distribution for gene location (45). We used the score statistic

corresponding to this likelihood and estimated the variance by Monte Carlo simulations using Merlin. We calculated the contribution of each family to the maximum LOD score with Merlin. To analyze whether families affected with similar joint sites contributed to the maximum LOD score, we calculated Pearson's correlation coefficient between the LOD score and hip, knee or hand OA families.

Association approaches. Joint linkage and association analysis was performed to identify SNPs that fully or partly explain the observed linkage signal using the program LAMP (http://csg.sph.umich.edu/LAMP) (15). The maximum likelihood was estimated using 50 starting points, and the disease prevalence was set at 0.01 because the ascertainment scheme of GARP showed that selection criteria were stringent (18). In the second more robust approach, we stratified all sibling pairs for IBD status and compared the IBD = 2 stratum with controls as reference group. To take into account uncertainty in the IBD status, in association analyses allele frequencies were weighted for the probability of sibling pairs to share alleles IBD. The IBD status was estimated for the location of each gene separately with the genotypes of microsatellite markers only using the IBD and grid (1 cM) option as implemented in Merlin (41). Haplotypes of individuals were estimated by the expectation maximization algorithm implemented in SNPHAP version 1.3 (http://www-gene.cimr.cam.ac.uk/clayton/software/). Haplotypes with posterior haplotype probabilities ≥ 0.5 were used in all subsequent analyses. Genotype and allele distributions in cases and controls were compared using standard χ^2 analysis-of-contingency tables. For the distribution of genotypes, Hardy Weinberg equilibrium was tested by using the HWE program of LINKUTIL (http://linkage.rockfeller.edu/ott/linkutil.htm). A logistic regression model was fitted to measure the strength of association, which is expressed as an OR with 95% confidence intervals and adjusted for age (years) and BMI (kg/m^2) in the Rotterdam control sample and GARP study. To adjust for family relationship among sibling pairs of the GARP study, standard errors were estimated from the variance between sibling pairs (robust standard errors) and used in each comparison between GARP subjects and controls (46). We performed robust standard error analyses using Stata SE8 software (Stata Corporation, USA). All other analyses were carried out with SPSS version 14 software (SPSS, Chicago, IL, USA). Meta-analyses were performed in R (http://www.r-project.org/). Summary ORs were estimated using the random-effects model of DerSimonian and Laird (47). The heterogeneity was quantified using the I^2 statistic for inconsistency (48) and its statistical significance was tested with the χ^2-distributed Cochran Q statistic (49). I^2 describes the proportion of variation that is unlikely to be due to chance and is considered large for values over 50% (48).Two-tailed P-values are reported for all analyses.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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