

ARTICLE

Strong linkage on 2q33.3 to familial early-onset generalized osteoarthritis and a consideration of two positional candidate genes

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A genomewide screen was performed in four extended families with early-onset generalized osteoarthritis (FOA) without dysplasia. The FOA phenotype within these families shows a dominant Mendelian inheritance pattern and may represent common osteoarthritis (OA) at later ages. An initial locus was confirmed by three additional families and refined by 14 markers to a two-point logarithm of odds score of 6.05 ($\theta = 0.00$) for marker *D2S155* at chromosome 2q33.3. This locus coincided with the highest multipoint nonparametric linkage score of 4.70 (P -value = 0.0013) at marker *D2S2358*. Haplotype analysis of family members delineated a narrow region with a number of possible positional candidates, of which we investigate here the two most likely ones: *PTHR2*, encoding parathyroid hormone receptor 2, and *FZD5*, encoding frizzled receptor 5. For *FZD5*, we did not observe a segregating variant, however, for *PTHR2*, a missense variant (A225S) cosegregated with FOA in one family. The frequency of the *PTHR2* variant was rare in a population-based sample, aged 55–70 years ($N = 1228$, 0.4%). Of the 11 carriers, 36% showed generalized radiographic OA as compared to 23% in the remaining population. None of the other families that contributed to the linkage revealed a segregating variant. Together, we have identified a locus on chromosome 2q33.3 for FOA. Candidate gene analysis suggested a possible association of a *PTHR2* variant with generalized radiographic OA; it is, however, unlikely the major disease gene for the observed linkage to the FOA phenotype.

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Introduction

Osteoarthritis (OA) is a degenerative disease of the joints characterized by degradation of the hyaline articular

cartilage and remodeling of the subchondral bone with sclerosis. Genetic factors play an important role in the etiology of the disease, especially for OA at the hand or hip, for disc degeneration (DD) and for OA at multiple sites.^{1,2} Segregation analysis of families with generalized OA indicated the presence of a major recessive OA gene with a residual multifactorial component.³ Together, it has become clear that different definitions of OA must be considered as distinct entities and may be caused by different (sets of) genes.⁴

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Genomewide linkage and association studies with various subtypes of OA, including hand, hip, knee or at multiple sites, have revealed linkage to multiple chromosome areas that may harbor OA genes.⁵ Successively to these linkage studies, new OA genes have emerged with variants that affected cartilage function.^{6–8} Investigations of these variants within other OA populations revealed confirmation in some studies;⁹ however, were excluded in others.^{10,11} Together, these studies highlight the complex and heterogeneous nature of OA genetic susceptibility. Additional genes and/or pathways may therefore be discovered to explain the genetic susceptibility to OA further.

A number of studies have focused on extended families with early-onset OA phenotypes accompanied by either mild spondyloepiphyseal dysplasia or multiple epiphyseal dysplasia. In these families, several mutations in genes encoding structural proteins of the extracellular matrix (ECM) have been identified.¹² Only few studies have focused on extended families with early-onset OA as primary disease process. Such families without dysplasia are difficult to identify; however, they may reveal major OA genes contributing to early-onset OA by relative severe mutations and to more common OA types at later ages through mild mutations. Moreover, such genes may provide insight into potential pathways not yet recognized to be involved in the onset of OA. Previously, we reported on a single early-onset family with familial generalized OA (FOA) without any dysplasia. None of the genes encoding the ECM structural proteins were found to be involved in the pathogenesis of this OA phenotype.¹³ In the current study, we performed a genome screen of this family and three additional early-onset generalized OA families. Replication of initial identified loci was performed with additional families and fine mapping of the most likely region of linkage with 14 additional markers. To investigate the role of two positional candidate genes that reside within the region of linkage, we undertook mutation analysis by genomic sequence and association analysis in a population-based cohort.

Subjects and methods

Ascertainment FOA families

Families containing patients that express primary generalized OA in each generation were collected from different parts of the Netherlands. Informed consent was obtained from all patients and the Medical Ethics Committee of the Leiden University Medical Centre approved the study. Probands were recognized through Rheumatology outpatient clinics. Family members were recruited via probands. Initially, we used questionnaires to select eligible families. For eligible families, complete medical history and available radiographs were obtained from the hospitals of almost all affected family members (81%). Radiographs

were re-evaluated for signs of chondrodysplasia, spinal dysplasia and abnormal development of the epiphyses of the peripheral joints. These features were absent in all families. The presence of radiographic characteristics of OA (ROA) was assessed according to Kellgren's criteria¹⁴ by an experienced reader (Dr C Bijkerk). Some individuals had marked Heberden's nodes and ankle OA. A selection of affected and unaffected individuals of families 1, 2, 4 and 7 were additionally visited for physical examination in order to prevent misclassification. The mean age of OA onset in these patients was 33 years and ranged between 20 and 50 years. The phenotype within these families is characterized by distinct progressive OA in the absence of mild or severe chondrodysplasia. Symptoms and ROA occur at multiple joint sites simultaneously, including involvement of the hands with noduli, knees, hips, ankle and spine. Individuals with clinical and radiographic evidence of OA in two or more joints before the age of 50 years were considered affected. Extensive description of the phenotype in family 1, which is representative for the phenotypes also of the other families included, is described elsewhere.¹³ All clinical diagnostic decisions were made independent to the genetic linkage analysis and homogeneity of the phenotype between different families was checked.

The Rotterdam sample

The Rotterdam study¹⁵ is a population-based cohort of in total 7983 Caucasian participants (response rate 78%). The medical ethics committee of the Erasmus University Medical School approved the study and written informed consent was obtained from each participant. In a random sample of 1369 unrelated subjects (the Rotterdam sample), aged 55–70 years, radiographs were scored for the presence of ROA in two knees, two hips,¹⁶ 36 hand joints and three levels of the thoracolumbar spine.² All radiographs were scored according to the Kellgren's criteria¹⁴ by two independent readers, blinded to all other data of the participant. Definite ROA was defined as a Kellgren's score of ≥ 2 .

In this study, we evaluated the association between generalized ROA and the *PTHR2* A225S variant in the Rotterdam sample using a qualitative and a quantitative ROA trait. The qualitative generalized ROA phenotype was described in detail elsewhere⁹ and occurred at a frequency of 23% in the Rotterdam sample. For the quantitative trait, an ROA score, based on the number of joints with ROA, was defined equivalent to the criteria described previously.¹⁷ In short, the specific ROA score (0–2) for the hip represented subjects with no (88%), unilateral (7%) or bilateral (5%) hip ROA. The knee ROA score represented subjects with no (81%), unilateral (11%) or bilateral (8%) knee ROA. For hand and spinal disk DD an ROA score proportional to the hip and knee was made. Hand ROA score (0–2) represented subjects with, respectively, 0 (40%), 1–2 (34%) and ≥ 3 hand joint groups affected

(26%). For spinal DD score (0–2) represents subjects with DD at, respectively, 0 (33%), 1 (38%) and ≥ 2 levels (30%). The quantitative total ROA score (0–8) was made by the sum of the proportioned joint-site-specific ROA scores and represents a quantitative measure of ROA within each patient. The mean and median total ROA score in the Rotterdam sample was 2.3 and 2.0, respectively.

Genotype analysis

For the initial genomewide scan, 231 microsatellite markers, with an average spacing of 18 cM, were used which were selected from the Cooperative Human Linkage Center (CHLC) human screening set (Weber version 8.0). Fine mapping was performed using 14 additional markers in the region of positive linkage, which were selected from the Genome Database and the Marshfield Medical Research Foundation <http://research.marshfieldclinic.org/genetics/>. The fluorescent-labeled PCR products were electrophoretically separated with automated laser fluorescence DNA sequencer (Pharmacia Biotec). Alleles were identified with Fragment analyzer (Pharmacia Biotech). The *PTHR2* A225S variant was genotyped in the Rotterdam sample by using Sequenom homogenous MassExtend Massarray System (Sequenom Inc., San Diego, CA, USA) using standard conditions. Genotypes were analyzed using Genotyper version 3.0 software (Sequenom Inc.). Successful genotypes were obtained for 1228 samples.

Linkage analysis

Family members are affected at relatively early age in multiple joint sites simultaneously, which enabled us to give a good estimation of the parameters that describe the mode of trait inheritance. Model-based linkage analysis was performed using the FASTLINK 2.2 version of the linkage program MLINK.¹⁸ The disease locus was modeled as an autosomal-dominant trait with a disease frequency of 0.001, as symptomatic and radiographic OA at multiple joint sites before the age of 50 years is rare. Marker allele frequencies were used from the CEPH database and compared with data of the founders. Penetrances were assumed to rise linearly from 0% at age 15 to 100% at age 50 with a phenocopy penetrance of 0.001. Model-based linkage analysis procedures can handle large pedigrees, but only up to one or two loci at a time.

Multipoint, model-free linkage analysis was performed and haplotypes were constructed using GENEHUNTER2.¹⁹ The Genehunter software does not require assumptions related to mode of inheritance or penetrance and it examines whether the allele sharing among affected relatives is greater than expected under the null hypothesis. The program can handle large numbers of loci but only on a small number of family members at a time.

Linkage analysis strategy

We used a stepwise linkage analysis strategy. The first step was to genotype an 18-cM map of markers using four original FOA families. Any marker that showed a two-point LOD score of ≥ 1 in the parametric linkage analysis was indicated as initial linkage signal. The second step was to replicate the initial linkage result using three additional FOA families that were ascertained during the genotyping of the whole-genome scan. The third step consisted of refinement of the most promising linkage result by fine mapping analysis in all available FOA families. Finally, multipoint model-free linkage analyses were applied to increase the marker informativity and to check the robustness of the observed model based linkage.

Sequence analysis

Genomic DNA was used as template to generate PCR products for sequence reactions. Primers sequences were chosen to produce fragments that contained a single exon and at least 60 bp of each flanking intron sequence. For the *PTHR2* gene (NCBI Genbank accession number, NM_005048), exons and flanking intron sequences, containing the complete coding region, the core promoter and part of the untranslated sequences, were screened for the presence of mutations by sequence analysis of both affected and unaffected individuals. For *FZD5* (NCBI Genbank accession number NM_003468), we sequenced the two exons. Sequencing of PCR products was performed with ABI PRISM[®] 3700 DNA analyzer (Applied Biosystems) using a sequencing kit (Applied Biosystems). The NCBI SNP database (build 124) was checked for the presence of reference SNP codes for variants observed (<http://www.ncbi.nlm.nih.gov/SNP/>). PolyPhen was used to investigate the possible impact of a nonsynonymous base pair change on the structure and function. The prediction of PolyPhen is based on physical and comparative considerations <http://tux.embl-heidelberg.de/ramensky/>.²⁰

Occurrence of *PTHR2* mutations and association with generalized OA

The *PTHR2* A225S variant was evaluated in the Rotterdam sample ($N=1228$) based both on its frequency and its occurrence with a generalized OA status (see above). Hardy–Weinberg equilibrium (HWE) was calculated with an exact HWE test for rare alleles implemented in R version 1.9.1 (<http://www.r-project.org/>). A logistic regression model was fitted to measure the strength of association with the qualitative generalized OA trait, which is expressed as odds ratios (OR) with 95% confidence intervals (95% CI) adjusted for age (years), body mass index (BMI, in kg/m^2) and sex. Furthermore, differences of the quantitative ROA score between carriers and noncarriers were determined by using an independent *t*-test and the nonparametric Mann–Whitney *U*-test. Homozygous carriers of the risk allele were not observed. All analyses

were performed with SPSS version 11 software (SPSS, Chicago, IL, USA).

Results

Genomewide linkage scan and replication of positive linkage signals

In the genome scan, we studied four FOA families (families 1–4) with generalized OA in each generation (Figure 1). Two-point model-based linkage analysis revealed two loci on chromosome 2, *D2S1334* (2q22.1–22.3) and *D2S1391* (2q32) with an LOD score above 1. None of the other markers throughout the genome provided a likely location for an OA gene in our FOA families. Three additional FOA

families, which were ascertained during the genotypings of genome scan of the first four families (Figure 1; families 5–7), were used to replicate the initial regions of linkage. For the 2q22.1–22.3 region, no sustained evidence of linkage emerged.

Fine mapping analysis

For the region surrounding marker *D2S1391* (2q32), a selection of 14 additional markers were genotyped in all families to provide denser coverage. The highest two-point LOD score was found for marker *D2S155* on chromosome locus 2q33.3 of 6.05 at recombination fraction 0.00. As shown in Table 1, families 1 and 2 contribute significantly to the height, whereas family 7 contributes negatively to

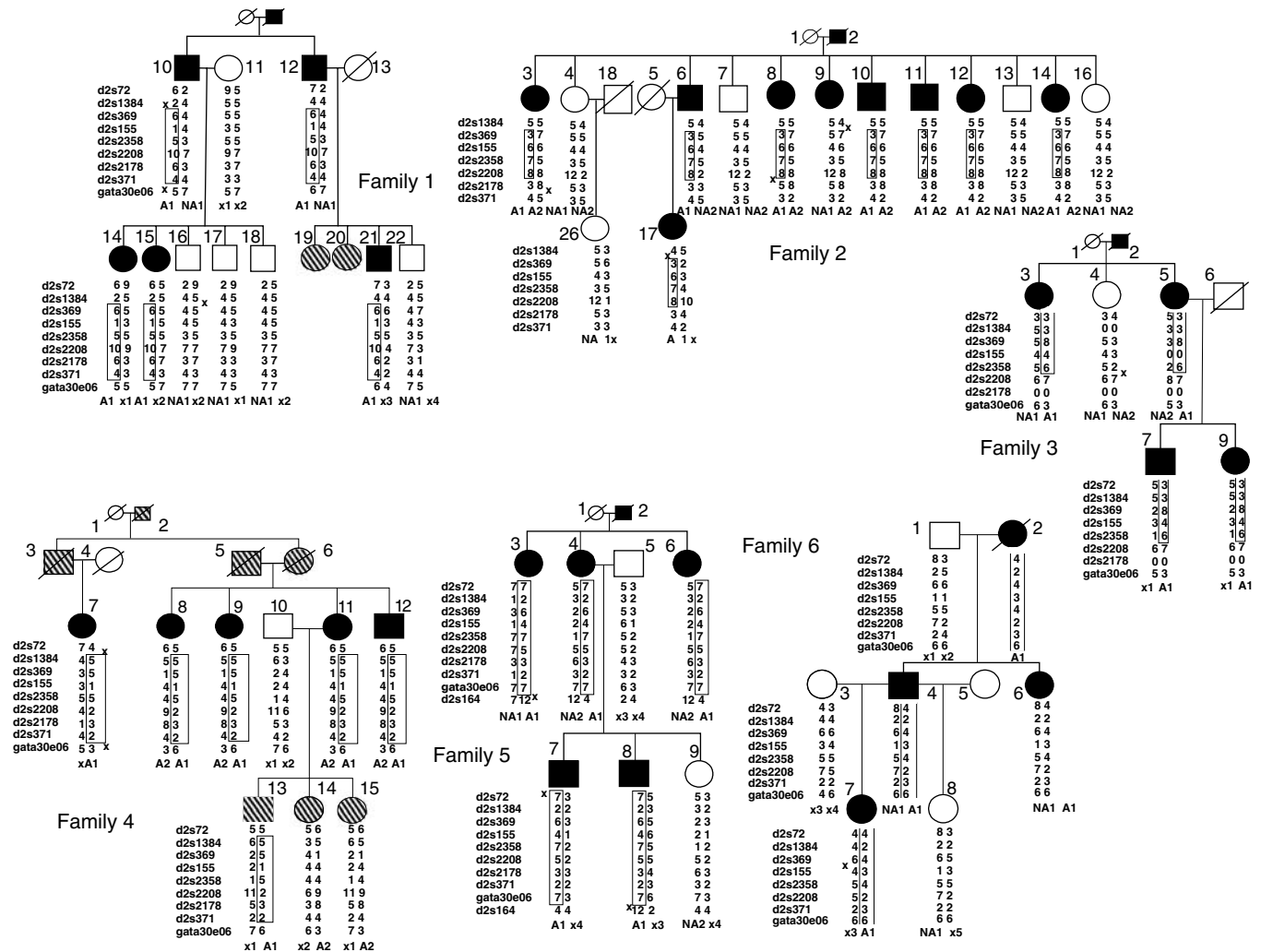


Figure 1 Pedigree structures of the FOA families. Families 1–4 were used for the initial genomewide scan, additional families for replication analysis. Circles denote females and squares denote males. Blackened symbols are affected individuals and symbols with diagonal lines represent diagnostic uncertainty. The cosegregating haplotypes among affected individuals are depicted as squared boxes, recombination's as crosses. Boxes that are open at the top or bottom continue. A1 is most likely affected haplotype and A2 second likely haplotype. NA1 most likely unaffected haplotype and NA2 second most likely haplotype. Family 7 is not added to the figure as it did not contribute to the linkage.

this LOD score. Multipoint analysis of this region showed nonparametric linkage (NPL) scores above 3 for the region between markers *D2S1384* and *D2S371* (6 cM). The highest multipoint NPL score was established for marker *D2S2358* of 4.70 (Figure 2; *P*-value 0.0013).

Recombinant haplotypes were determined for all families that contributed positively to the linkage. In families 2 and 4, however, two haplotypes are shared among affected individuals, which are absent in the unaffected relatives resulting in two possible haplotypes that contribute to the linkage (Figure 1). For example, in family 2, the most likely haplotype with alleles 3-6-7-8 (A1) allocates one phenocopy (individual 9), whereas the second likely haplotype 7-6-5-8 (A2) allocates two phenocopies (subjects 6 and 17). In family 4, the most likely haplotype (A1) allocates no phenocopies and the second

likely haplotype (A2) allows one phenocopy (individual 7). As also shown in Figure 1, alleles determining cosegregating haplotypes were not identical among families, indicating that different causal variants may exist contributing to the onset of the FOA phenotype. The candidate gene region was refined by combining the cosegregating haplotypes among affected individuals of families 1 and 2, as they contributed most to the linkage. A minimal candidate gene region was defined between the markers *D2S1384* and *D2S2178* with a distance of 5 cM, 4.6 Mb (ENSEMBL database v35). This region completely encompassed the cosegregating haplotype of family 4.

Candidate genes

A query of the human genome resources within the region on chromosome 2q33.3 between markers *D2S1384* and *D2S2178* disclosed 18 known genes, and nine predicted RefSeq genes (NCBI build 35; Supplementary Table 1). Concerning the tissue expression pattern, function, functional domains and previous studies on these proteins, two genes, *FZD5* and *PTHR2*, emerged as functional candidates to be involved in the onset of generalized OA.

Mutation analysis

Sequence analysis was performed to identify potential DNA variations in affected and unaffected members of the OA families. In the two exons of *FZD5* gene, we did not observe a variant, which cosegregated with the OA phenotypes in the families. For the *PTHR2* gene, we identified three known SNPs, a rare nonsense variant in exon 3 and a rare missense variant in exon 6, respectively. The nonsense variant (c.458C>A, p.S82X) located in the first extracellular domain of the *PTHR2*, however, was

Table 1 Two-point parametric linkage analysis of chromosome 2q marker *D2S155* using seven families with early onset generalized osteoarthritis

Marker name	cM ^a	Thetas				
		0.0	0.01	0.05	0.10	0.15
D2S155	202.9					
Overall		6.05	5.96	5.55	4.96	4.32
Family 1		1.81	1.77	1.62	1.44	1.25
Family 2		2.93	2.88	2.67	2.39	2.11
Family 3		0.26	0.25	0.22	0.18	0.15
Family 4		0.35	0.34	0.30	0.26	0.21
Family 5		0.51	0.50	0.46	0.40	0.35
Family 6		0.76	0.75	0.67	0.56	0.46
Family 7		-0.58	-0.53	-0.39	-0.28	0.19

^aMarshfield sex average linkage map.

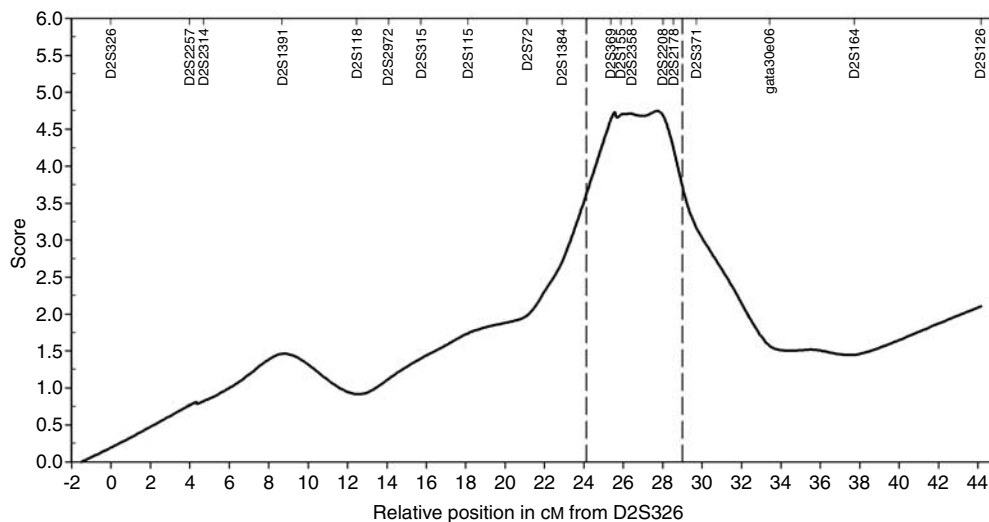


Figure 2 Multipoint model-free NPL scores of 19 markers covering the 44 cM region on chromosome 2q33.3 using all families. cM are provided relative to marker *D2S326*, which is located at the centromeric edge of the region of linkage. The dashed vertical lines indicate the one-LOD-drop interval.

present in all unaffected subjects of family 2 (haplotype NA1) and does, therefore, not segregate with the disease phenotype. The missense variant (c.786G>T, p.A225S) was located within the second extracellular domain of the gene and was found on the second likely haplotype A2 of family 4. As a result, the more distant affected subject number 7 in family 4 (niece) did not carry the mutation. The mutation, therefore, may be causal to the FOA phenotype only when the distant relative is a phenocopy. The nonsynonymous change was predicted to be benign by the PolyPhen software.²⁰

Association analysis

Screening of this mutation in a population-based Rotterdam sample with subjects aged between 55 and 70 years ($N=1228$) revealed 11 heterozygous individuals (allele frequency 0.004). This genotype distribution is in accordance with the HWE. In addition, four of the 11 carriers (36%) showed generalized ROA as compared to 23% in the population cohort, conferring to an odds ratio, adjusted for age, BMI and sex of 1.7, 95% CI 0.5–6.1. Furthermore, an increased mean and median number of joint sites with ROA as expressed by the total ROA score was observed for carriers (mean 3.1 and median 3.0, respectively) as compared to noncarriers (mean 2.3 and median 2.0, respectively) with P -value = 0.08 (t -test) and P -value = 0.10 (Mann–Whitney U -test).

Discussion

A genomewide scan was performed in families with dominant Mendelian inherited generalized OA. In these families, symptomatic OA occurs without detectable dysplasia at multiple joint sites simultaneously. Radiographically, the phenotype resembles primary OA occurring at later ages in the population, except for the early age of onset (20–50 years) and the progressive course of the disease. A new and narrow location was identified with a significant two-point model-based LOD score of 6.05 (theta 0.00) for marker *D2S155* on chromosome 2q33.3. Multipoint model-free linkage analysis was applied to increase the marker informativity and to check the robustness of the observed model based linkage and showed a maximal NPL score of 4.70 close to marker *D2S155* (P -value 0.0013; Figure 2). Although the increased marker information provided by multipoint analyses could have resulted in higher NPL scores, the increase in marker informativity may have been nullified by the smaller number of family members that could be analyzed by the GENEHUNTER2 program. Subsequent sequence analysis of two promising candidate genes revealed a missense variant in the *PTHR2* gene that cosegregate with FOA in one family.

Extended families and model-based linkage analysis may provide high power to identify linkage, especially when the correct model is used. In the current analyses, we used a

dominant inheritance pattern. In two families (families 2 and 4), however, the majority of the affected family members shared two haplotypes that may indicate a recessive mode of inheritance. Especially for those two families, the dominant model used may have resulted in a suboptimal linkage result.

Up until now, there is no widely accepted definition of generalized OA. In epidemiological studies, the term is used to describe subjects with 2–3 radiographically affected sites, which may occur relatively frequent at ages above 55 years when considering, for example, hand and spine.² In contrast, the diagnosis of FOA in our families reflect patients with early onset symptomatic polyarticular disease, affecting typical as well as atypical sites and occurs in an autosomal-dominant inheritance patterns.¹³ Such a specific phenotype is rare, however, emerged after radiographic and physical examination as homogeneous among our family members. In the linkage analysis, we also assumed phenotypic and locus homogeneity among the families. Consequently, family 7 contributed negatively to the LOD score and may actually represent locus heterogeneity in the onset of FOA. Furthermore, although the linkage of the remaining smaller families (families 4, 5 and 6) was positive and almost maximal in each case, they provided no significant proof by themselves (Table 1). To minimize the risk of misclassification and missing out on important genes localized just outside the minimal cosegregating haplotype determined by all families together, we used recombinations of the two largest families to determine the candidate gene region. These recombinations appear to be of affected individuals.

Two independent genomewide searches were performed previously, of nodal OA²¹ and hip replacement²² that showed linkage to chromosome 2q32 encompassing the locus identified in our initial analyses. Two functional variants within the *FRZB* gene was subsequently found that explained the linkage and were associated to hip replacement in females,⁶ signifying the Wnt signaling pathway in the pathogenesis of OA. Upon fine mapping analysis, our locus shifted from the 2q32 toward the telomere, thereby discarding the overlap with the previously observed linkage in this region.^{21,22} Two promising candidate genes for the FOA phenotype, *PTHR2* and the *FZD5*, mapped within the minimal cosegregating haplotype. The *FZD5* gene encodes the Wnt signaling receptor FZ5 for the Wnt5A ligand²³ and might agonist FRZB. Detectable levels of *FZD5* mRNA were present in OA tissue and the ligand receptor pair is implicated to be essential in cell fate determination and OA.⁶ We sequenced the *FZD5* gene from transcription start site until the stop codon and did not observe a variant cosegregating with affected family members. Alternatively, affected family members may have a noncoding regulatory mutation in promoter or intron.

The parathyroid hormone 2 (PTH2) receptor is a G-protein-coupled receptor selectively activated with similar

potency by PTH and tuberoinfundibular peptide (TIP39).²⁴ Relevant functions for its possible role in OA are the expression of *PTHR2* in a number of endocrine cell types that suggest a role for the PTH receptor 2 in regulating pituitary hormone secretion and specifically growth hormone. Moreover, in peripheral organs distinct cell populations express PTH receptor 2, including the calcitonin-synthesizing parafollicular C cells of the thyroid gland involved in Ca^{2+} homeostasis and chondrocytes within cartilage growth plates of developing bone.²⁴ Recently, the PTH receptor 2 has been shown to interact with calmodulin1 (CaM1), a calcium-sensing protein,²⁵ whereas a functional SNP in the core promoter region of the gene encoding calmodulin1 (*CALM1*), was associated with hip OA in the Japanese population.⁸ Although the association with this functional SNP was not replicated in a UK population,¹⁰ the CaM1-mediated signaling pathway is considered to be a new paradigm in the etiology and pathogenesis of OA.²⁶ Together, further research is necessary to explore the role of the PTH receptor 2 in the onset of OA.

In the *PTHR2* gene, a missense mutation was identified cosegregating with the affection status in family 4 (Figure 1). The variant was predicted to be benign by the PolyPhen software; however, it appeared to be very rare in a 55- to 70-year-old population-based sample from the Rotterdam sample ($N=1228$, frequency 0.004) and was possibly associated toward generalized radiographic OA with an OR of 1.7, 95% CI 0.5–6.1. The low frequency of the allele, however, hampered a powerful association analysis also reflected by the large CI limits. Furthermore, owing to the fact that some carriers in the population exist with relative mild ROA, the A225S variant can only be considered causal when variable penetrance is assumed or when additional modulators exist in the FOA family. In order to explore the role of this variant further, it is necessary to validate or confirm this association in another early-onset generalized FOA disease population. Together, our data indicate that the *PTHR2* and/or the *FZD5* genes are unlikely the major disease gene for the observed linkage to the FOA phenotype. Mutation survey of other genes irrespective of their function in the chromosomal area is ongoing.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)