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Deep sequencing of GDF5 reveals the absence of rare variants at this important osteoarthritis susceptibility locus

A.W. Dodd †[a], C. Rodriguez-Fontenla ‡[a], M. Calaza ‡, A. Carr §, J.J. Gomez-Reino ‡, A. Tsezou ||, L.N. Reynard †, A. Gonzalez ‡, J. Loughlin †*

† Newcastle University, Institute of Cellular Medicine, Newcastle, UK
‡ Instituto Investigacion Sanitaria, Hospital Clinico Universitario de Santiago, Santiago de Compostela, Spain
§ University of Oxford, NDORMS, Oxford, UK
|| University of Thessaly, Medical School, Department of Biology, Larissa, Greece

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SUMMARY

Objective: The common single nucleotide polymorphism (SNP) rs143383 in the 5′ untranslated region (5′UTR) of growth and differentiation factor 5 (*GDF5*) is strongly associated with osteoarthritis (OA) and influences *GDF5* allelic expression *in vitro* and in the joint tissues of OA patients. This effect is modulated in *cis* by another common SNP, also located within the 5′UTR, whilst a common SNP in the 3′UTR influences allelic expression independent of rs143383. DNA variants can be common, rare or extremely rare/unique. To therefore enhance our understanding of the allelic architecture of this very important OA susceptibility locus we sequenced the gene for potentially functional and novel rare variants.
Method: Using the Sanger method we sequenced *GDF5* in 992 OA patients and 944 controls, with DNA changes identified by sequencing software. We encompassed the protein-coding region of the two *GDF5* exons, both untranslated regions and approximately 100 bp of the proximal promoter of the gene.
Results: We detected 13 variants. Six were extremely rare with minor allele frequencies (MAFs) of $\leq$0.0006. One is in a predicted transcription factor binding site in the *GDF5* promoter whilst two substitute conserved amino acids. The remaining seven variants were common and are previously known variants, with MAFs ranging from 0.025 to 0.39. There was a complete absence of variants with frequencies in-between the extremely rare ($n = 6$) and the common ($n = 7$).
Conclusions: This is the first report of the deep sequencing of an OA susceptibility locus. The absence of rare variants informs us that within the regions of the gene that we have sequenced *GDF5* does not harbour any novel variants that are able to contribute, at a population level, to the OA association signal mediated by rs143383 nor does it harbour, at a population level, any novel variants that can influence OA susceptibility independent of rs143383.

Introduction

Growth and differentiation factor 5 (GDF5), also known as cartilage derived morphogenetic protein 1 (CDMP1), is an extracellular signalling molecule that participates in the development, maintenance and repair of bone, cartilage and other tissues of the synovial joint[1], with penetrant and rare deleterious mutations of the *GDF5* gene resulting in dominant skeletal defects[2]. Based on this functional knowledge the *GDF5* gene was chosen by a Japanese group for examination as a potential osteoarthritis (OA) susceptibility locus[3]. A number of common *GDF5* polymorphisms were genotyped in cases and in controls and association to the common single nucleotide polymorphism (SNP) rs143383, a T to C transition located in the 5′ untranslated region (5′UTR) of the gene, was observed. In subsequent studies association of this SNP with OA was observed in Europeans, demonstrating that the association has global relevance[4–6]. Functional studies have suggested that rs143383 is itself the polymorphism influencing OA susceptibility with the OA-associated T-allele mediating reduced *GDF5* transcription relative to the C-allele[7,8]. Furthermore, association of rs143383 to other musculoskeletal phenotypes, including variation in normal height, Achilles tendon pathology, fracture risk and congenital dysplasia of the hip, has been reported. This highlights

* Address correspondence and reprint requests to: John Loughlin, Newcastle University, Institute of Cellular Medicine, Musculoskeletal Research Group, 4th Floor, Catherine Cookson Building, The Medical School, Framlington Place, Newcastle upon Tyne NE2 4HH, UK.
E-mail address: john.loughlin@ncl.ac.uk (J. Loughlin).
[a] These authors contributed equally to this work.

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the tendency of a common genotype to influence multiple phenotypes (pleiotropy) and of the potential role that developmental factors can have on conditions that manifest in the mature individual[9–13].

The negative effect of the OA-associated T-allele of rs143383 on *GDF5* transcription is modulated by a second common polymorphism located in the 5′UTR of the gene, rs143384; in a luciferase assay using chondrogenic cells it was demonstrated that the T-allele of rs143383 mediates a reduction in luciferase activity when it is on the background of a T-allele of rs143384 but not when it is on the background of the C-allele of this SNP[8]. Furthermore, using RNA extracted from the joint tissues of OA patients, this study demonstrated that a third SNP in *GDF5*, rs56366915 (referred to as 2250ct in ref[7]), which is located in the 3′UTR, affects allelic expression of *GDF5* independent of rs143383.

Mouse models have further developed our understanding of the role of GDF5 in skeletogenesis and joint maintenance. The brachypodism mouse is homozygote for a frame-shift mutation of *Gdf5* that causes a premature termination codon resulting in a null allele[14]. These mice have a number of abnormalities including a shortened appendicular skeleton, a decrease in the length of the limb long bones, and soft tissue deformities that clearly demonstrate the important role of GDF5 in normal development[14–16]. A haplo-insufficient mouse carrying one copy of the brachypodism allele does not show such developmental abnormalities but does show an increased propensity of developing an OA phenotype when challenged[17]. This model, which from a molecular genetics perspective mimics the effect of SNP rs143383 in human OA, confirms the importance of maintaining adequate levels of GDF5 for normal joint function and emphasizes again the detrimental effects of quantitative alterations in its expression on joint tissue maintenance.

Overall, the rs143383 polymorphism is a very significant contributor to OA susceptibility, albeit with modest effect sizes (odds ratios < 1.2). This polymorphism is functionally complex, with the allelic expression mediated by rs143383 being modulated by another common SNP and with a third SNP influencing allelic expression in an independent manner. Cataloguing the variation at this gene is therefore critical to our full understanding of this important locus and we set out to assess the allelic architecture of *GDF5* in OA populations from northern and southern Europe using a deep-sequencing approach.

Patients and methods

Cases and controls

OA cases and controls were studied from the UK, Spain and Greece. All were of Caucasian ethnicity. The UK cohort were north Europeans of white British origin, the Spanish cohort were Europeans of Spanish origin and the Greek cohort were Europeans of central Greek origin. There were 992 cases that had each undergone a total knee or a total hip joint replacement due to severe primary OA and 944 controls that lacked clinical signs of the disease. Comprehensive details about the cases and the controls have been published[7,18].

In the UK cohort there were 502 cases (383 females and 119 males; 220 knee cases and 282 hip) and 460 controls (184 females and 276 males). The cases were ascertained using the criteria of signs and symptoms of OA sufficiently severe to require joint replacement surgery. The radiological stage of the disease was a Kellgren and Lawrence 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. The cases had an age range of 56–85 years. The controls had no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness or restriction of movement) and had an age range of 55–89 years.

In the Spanish cohort there were 264 cases (214 females and 50 males, all knee cases) and 294 controls (207 females and 87 males). Cases were selected as consecutive patients, aged 55–80 years of age at the time of surgery, undergoing total knee replacement if a rheumatologist considered them to suffer from severe primary OA. Exclusion criteria were inflammatory, infectious, traumatic or congenital joint pathology and lesions due to crystal deposition or osteonecrosis. Controls were recruited among subjects older than 55 years of age undergoing preoperative work-up for elective surgeries other than joint surgery and who did not show clinical manifestations of OA.

In the Greek cohort there were 226 cases (200 females and 26 males, all knee cases) and 190 controls (137 females and 53 males). The cases had a Kellgren and Lawrence grade of greater than 2 prior to total knee replacement and were randomly selected. Individuals with rheumatoid arthritis and other autoimmune diseases as well as chondrodysplasias, infection-induced OA, and post-traumatic OA were excluded. The cases had an age range of 40–92 years. The controls had undergone treatment for injuries and fractures and had an age range of 46–88 years. All controls had a Kellgren and Lawrence grade of 0.

Ethical approvals for the use of the DNAs in OA genetic studies were obtained from local ethical committees: Oxford for the UK cohort, Santiago for the Spanish cohort and Larissa for the Greek cohort.

DNA sequencing

Using 50 ng of DNA extracted from peripheral blood samples by phenol–chloroform or guanidine hydrochloride *GDF5* was polymerase chain reaction (PCR) amplified with overlapping amplimers and these were then subjected to dideoxy Sanger sequencing using the BigDye v3.1 kit (Applied Biosystems) and 3130 Genetic Analyzer capillary sequencers (Applied Biosystems). In the Spanish and Greek cohorts the search for variants focused on the protein-coding regions of the two exons of *GDF5* and the intron/exon flanking sequences, whilst the UK search encompassed the protein-coding regions, the intron/exon flanking sequences, both UTRs and 100 bp of the proximal promoter of *GDF5* (Fig. 1A). The sequences of the primers used are listed in Supplementary Table I. The Polyphred and SeqScape software's (Applied Biosystems) were used to align sequences and to identify DNA changes. Novel variants were confirmed by designing a new PCR amplimer and repeating the sequencing on a fresh aliquot of DNA.

Variant 1 was genotyped in the Spanish and Greek cohorts using the SNaPshot minisequencing protocol (Applied Biosystems) and the PCR forward primer 5′TTCCGTTTCCAATTCCTGAG3′, the PCR reverse primer 5′TGACTGAGGGCTTGAAGGAG3′ and the SNaPshot primer 5′AACTAGGGGGAAAAAAAAACTGGAGCACACAAGCAGCATTACGCCATT3′.

Results

Known variants

GDF5 is 4.9 kb in length and contains two exons coding for a 510 amino acid protein. We identified seven common variants that were previously known to exist in the gene: rs143383, rs73094730, rs143384, rs224331, rs224330, rs73611720 and rs56366915 (Fig. 1B, Supplementary Table II). rs224331 and rs224330 were observed in the UK, Spanish and Greek cohorts, since the region of *GDF5* harbouring these two variants (exon 2) was encompassed in the

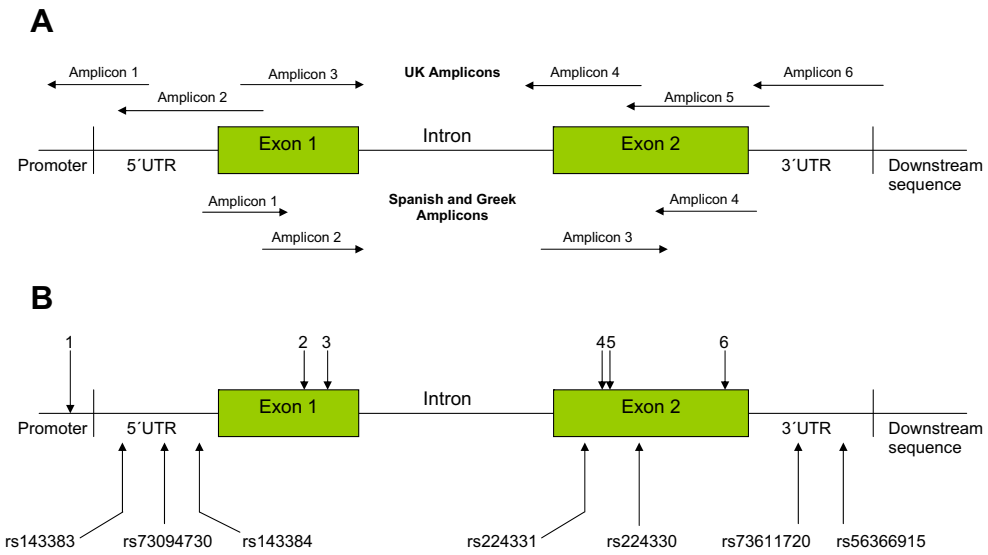

Fig. 1. The sections of *GDF5* sequenced and the location of the newly discovered variants. (A) The six amplicons used for the sequencing in the UK cohort and the four amplicons used for the Spanish and Greek cohorts. (B) At the top the arrows point to the approximate location of each of the six novel variants. At the bottom the arrows point to the approximate location of the seven known variants that are present in dbSNP and which we confirmed.

sequencing of all three of these cohorts (Fig. 1A). The remaining five variants were observed only in the UK cohort, since the regions harbouring these five variants (the 5′ and 3′ UTRs) were only sequenced in this cohort (Fig. 1A).

None of the known variants identified by us demonstrated association to OA ($P < 0.05$), with association analysis performed unstratified and stratified by cohort, by sex and by joint (data not shown). We were not particularly surprised by this result since it is known that large sample sizes are required to generate robust association to rs143383[4–6] and by comparison our sample size is small and underpowered. Furthermore, and as noted above, rs224331 and rs224330 were the only SNPs with data available in all three cohorts, thus reducing further any power for the five variants with genotype data available only from the UK cohort.

There are several other *GDF5* SNPs listed in dbSNP but these either lack frequency data or have so far been shown to be polymorphic only in non-European samples. These are rs34534075, rs61754581, rs28936397, rs76603468, rs61754580, rs28936683, rs17853055, rs5841206, rs6120942 and rs79051206 (http://www.ncbi.nlm.nih.gov/projects/SNP/). We did not detect these variants despite their coverage in our sequence analysis and we conclude therefore that they are not polymorphic in Europeans, at least in the large sample that we have studied.

Novel variants

We identified six novel and extremely rare variants, all in the UK cohort (Fig. 1, Table I). Each variant was detected in a single individual, two in cases and four in controls. The six UK individuals carrying a variant allele were each heterozygote for the variant allele that they carried. Variant 1 was discovered in a female control following the successful sequencing of 458 cases and 418 controls and therefore has a MAF in the UK sample of 0.0006 (1 mutant allele in 1752 chromosomes). Variants 2 (female case with knee OA) and 3 (male case with knee OA) were discovered following the successful sequencing of 491 cases and 425 controls and therefore have minor allele frequencies (MAFs) in the UK sample of 0.0005 (1 mutant allele of each variant in 1832 chromosomes). Variants 4 (male control) and 5 (female control) were discovered following the successful sequencing of 474 cases and 389 controls and therefore have MAFs in the UK sample of 0.0006 (1 mutant allele of each variant in 1726 chromosomes). Variant 6 (female control) was discovered following the successful sequencing of 495 cases and 436 controls and therefore has a MAF in the UK sample of 0.0005 (1 mutant allele in 1862 chromosomes). For variants 2–6 these MAFs are upper-bound estimates, as the variants were not observed in the Spanish or Greek samples. The region of *GDF5* harbouring variant 1 was not included in the sequence analysis of the Spanish and Greek cohorts. We therefore directly genotyped this variant in these cohorts, using as a positive the UK female carrying the variant. The variant was not detected in the Spanish or Greek cohorts. Variant 1, like variants 2–6, was therefore observed only in the UK cohort.

We then used bioinformatics tools to see if any of these novel variants had the potential to alter the GDF5 protein or affect transcription factor binding to the *GDF5* gene. Variant 1 is located in the promoter of *GDF5*, 41 bp upstream of the transcription initiation site. Using the transcription factor binding profile databases Jaspar (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl), TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html) and Alggen

Table I
The six novel variants detected in *GDF5*. Each variant was detected in single individuals from the UK. The variants were not observed in the Spanish or in the Greek cohorts

Variant	Individual	Wild-type allele	Mutant allele	Location	Predicted effect
1	Control	C	A	Promoter	Alteration of a SOX9 binding site
2	Case	G	A	Exon 1	Synonymous, Gly67Gly
3	Case	G	A	Exon 1	Non-synonymous, Gly81Arg
4	Control	C	T	Exon 2	Synonymous, Gly285Gly
5	Control	C	T	Exon 2	Synonymous, Asp287Asp
6	Control	C	G	Exon 2	Non-synonymous, Thr469Arg

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Homo sapiens Gly81Arg      PPLARN-VFRPGGHSYGGGATNANARAKGGTRQTGGLTQPKKDEPKKLPP 99
Homo sapiens               PPLARN-VFRPGGHSYGGGATNANARAKGGTGQTGGLTQPKKDEPKKLPP 99
Pan troglodytes            PPLARN-VFRPGGHSYGGGATNANARAKGGTGQKGGLTQPKKDEPKKLPP 99
Equus caballus             PPLARN-IFRPGGHSYGGGAT--SARAKGGTGQTGGPTQPKKDEPKKLPP 97
Mus musculus               PPLARN-VFRPGGHIYGVGAT--NARAKGSSGQ----TQAKKDEPRKMPP 93
Gallus gallus              TASARAGAPRAANHGFSTGTS--KARAKSNAAQAG-ALLAKNDESQRVLS 97
Xenopus tropicalis         NPLPKVNATRTGILGHGVGLQ--KGRSKVPLVQSR-IFLSKNEDIKKQAA 95

Homo sapiens Thr469Arg     HAVIQTLMNSMDPESTPPTCCVPRRLSPISILFIDSANNVVYKQYEDMVV 495
Homo sapiens               HAVIQTLMNSMDPESTPPTCCVPTRLSPISILFIDSANNVVYKQYEDMVV 495
Pan troglodytes            HAVIQTLMNSMDPESTPPTCCVPTRLSPISILFIDSANNVVYKQYEDMVV 495
Equus caballus             HAVIQTLMNSMDPESTPPTCCVPTRLSPISILFIDSANNVVYKQYEDMVV 493
Mus musculus               HAVIQTLMNSMDPESTPPTCCVPTRLSPISILFIDSANNVVYKQYEDMVV 489
Gallus gallus              HAVIQTLMNSMDPESTPPTCCVPTRLSPISILFIDSANNVVYKQYEDMVV 494
Xenopus tropicalis         HAVIQTLMNSMDPETTPPTCCVPTRLSPISILYTDSANNVVYKQYEDMVV 490
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Fig. 2. The amino acid sequence alignments for variants 3 and 6. The amino acid sequences of variant 3, Gly81Arg (top, highlighted in blue), and of variant 6, Thr469Arg (bottom, highlighted in red), were aligned with human wild-type GDF5 and with GDF5 of other species.

(http://alggen.lsi.upc.es/) we ascertained that the variant was predicted to abolish HSF and CAP binding sites, to create CdxA and Ubx binding sites and to alter SOX9, SOX10 and NFATC2 binding sites.

The glycine to arginine substitution coded for by variant 3 is at a residue conserved in mammals but not in vertebrates (Fig. 2). The substitution was predicted to be benign by the PolyPhen database, which predicts the functional effect of non-synonymous human SNPs (http://genetics.bwh.harvard.edu/pph/). The threonine to arginine substitution coded for by variant 6 is in the mature part of the protein at a highly conserved residue, conserved in mammals and other vertebrates. PolyPhen predicted this substitution to be potentially damaging.

Absence of rare variants

Our sequencing of *GDF5* did not detect any rare variants, that is variants with a MAF in the region of 0.001–0.025 (i.e., 0.1–2.5%). Instead, variants were either extremely rare (MAFs $\leq$ 0.0006, i.e., $\leq$0.06%, $n = 6$) or common (MAFs $>$ 0.025, i.e., 2.5%, $n = 7$), as represented in Fig. 3.

Discussion

It is becoming increasingly apparent that loci harbouring major susceptibility alleles for a particular trait are also likely to be

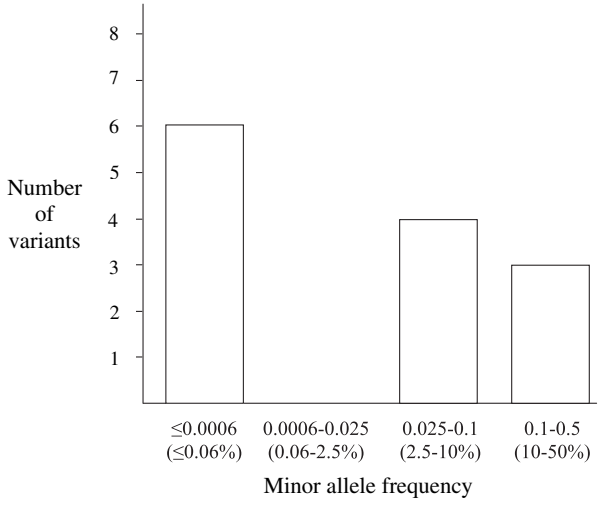

Fig. 3. The number of variants at *GDF5* displayed according to their MAFs in the UK cohort.

repositories for other alleles influencing that trait[19]. The growth and differentiation factor 5 gene *GDF5* contains a common functional SNP, rs143383, the T-allele of which is significantly associated with the development of OA and of other musculoskeletal phenotypes. From a previous study we had established that the effect of rs143383 on allelic expression imbalance of *GDF5* is influenced by genotype at a second 5′UTR SNP, rs143384, whilst the 3′UTR SNP rs56366915 correlates with allelic expression imbalance independent of rs143383[8]. rs143384 and rs56366915 are both common SNPs that are present in public databases. In this study we therefore set out to assess the allelic architecture of *GDF5* to assess whether this gene harboured other potentially functional variants that may be of relevance to OA aetiology. As far as we are aware this is the first example of a deep-sequencing analysis of an OA susceptibility locus.

We sequenced *GDF5* in cohorts of OA cases and non-OA controls from northern and southern Europe. This focus on cases and controls, rather than on individuals drawn from the general population, increased our likelihood of detecting penetrant risk or protective alleles. We demonstrated the accuracy of our sequencing by detecting all of the known variants from dbSNP that reside within the sections of the gene that we covered. We also demonstrated its sensitivity by identifying six extremely rare variants, present in single individuals from amongst the large cohort sequenced. Three of these extremely rare variants are potentially functional; variant 1, which is located in predicted transcription factor binding sites, variant 3, which codes for the substitution of a conserved glycine residue and variant 6, which codes for the substitution of a highly conserved threonine residue. Since these variants are extremely rare they cannot have an impact, at a population level, on OA susceptibility, on allelic expression imbalance mediated by rs143383 or on allelic expression imbalance independent of rs143383. If they are functional then they may have an effect at the individual and family level. Highly penetrant, deleterious mutations of *GDF5* can result in autosomal dominant brachydactyly, characterized by malformation of the phalanges[2]. Ethical policies with regards to genetic studies do not allow us to re-contact the UK individuals used in this study to discuss the impact of genetics on their health, so it is not possible for us to assess whether the individuals that harbour variant 1 (a control), variant 3 (a case) or variant 6 (a control) have a skeletal phenotype that was missed at their recruitment. Of the two non-synonymous variants that we detected, Gly81Arg was predicted to be benign by the PolyPhen database whilst Thr469Arg was predicted to be potentially damaging. Neither of these residues have been reported to be sites for brachydactyly mutations (http://www.hgmd.cf.ac.uk/ac/index.php).

In conclusion, our deep sequencing of the transcript sequence and promoter of *GDF5* has revealed that this gene harbours

extremely rare variants and that it harbours common variants but that there is an absence of variants of intermediate frequency. In Europeans there are therefore unlikely to be additional proximal variants of this locus that, at a population level, influence allelic expression imbalance of the gene and contribute to OA susceptibility. The currently known variants should be the target for future functional studies.

Role of the funding source

The funding bodies had no role in the design of the study, data collection, analysis and interpretation of the data, the writing of the manuscript or in the decision to submit the manuscript.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Prof. Loughlin had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Dodd, Rodriguez-Fontenla, Gonzalez, Loughlin.

Acquisition of data: Dodd, Rodriguez-Fontenla, Calaza, Carr, Gomez-Reino, Tsezou, Reynard, Gonzalez, Loughlin.

Analysis and interpretation of data: Dodd, Rodriguez-Fontenla, Reynard, Gonzalez, Loughlin.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Supplementary material

Supplementary data associated with this article can be found in online version at doi:10.1016/j.joca.2011.01.014.

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