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Authors
Lucas, Gavin JA
Mehta, Sarju G
Hocking, Lynne J
et al.

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Evaluation of the role of Valosin-containing protein in the pathogenesis of familial and sporadic Paget's disease of bone

Gavin J.A. Lucas[a], Sarju G. Mehta[b], Lynne J. Hocking[a], Tracy L. Stewart[a], Tim Cundy[c], Geoff C. Nicholson[d], John P. Walsh[e], William D. Fraser[f], Giles D.J. Watts[b], Stuart H. Ralston[g,*], Virginia E. Kimonis[b]

[a]*Department of Medicine and Therapeutics, University of Aberdeen, UK*
[b]*Division of Genetics, Children's Hospital Boston, Harvard Medical School, MA 02132, USA*
[c]*Department of Medicine, University of Auckland, New Zealand*
[d]*Department of Clinical and Biomedical Sciences, Barwon Health, Geelong Hospital, University of Melbourne, Australia*
[e]*Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Perth, Western Australia*
[f]*Department of Clinical Chemistry, Royal Liverpool University Hospital, UK*
[g]*Rheumatic Diseases Unit, University of Edinburgh, Western General Hospital, Edinburgh EH2 2XU, UK*

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Abstract

Paget's disease of bone (PDB) is a common metabolic bone disease of late onset with a strong genetic component. Rarely, PDB can occur as part of a syndrome in which the disease is accompanied by inclusion body myopathy and frontotemporal dementia (inclusion body myopathy, Paget's disease and frontotemporal dementia, IBMPFD). Recently, IBMPFD has been shown to be caused by mutations in Valosin-containing Protein (VCP), which is required for the proteasomal degradation of phosphorylated IκB-α, a necessary step in the activation of the transcription factor NF-κB. Here, we evaluated the role of VCP in the pathogenesis of typical PDB. We conducted mutation screening of VCP in 44 kindreds with familial Paget's disease recruited mainly through clinic referrals in the UK, Australia and New Zealand. We also performed an association study of VCP haplotypes in patients with PDB who did not have a family history of the disease (sporadic PDB). No mutations were found in VCP in three PDB families where there was evidence of allele sharing between affected subjects in the VCP critical region on chromosome 9p13. We failed to detect disease-associated mutations in any of the three exons previously reported to contain IBMPFD mutations in a further 41 PDB families. We found no evidence of allelic association between common VCP haplotypes in a case-control study of 179 sporadic PDB patients and 172 age- and sex-matched controls. Genetic variation in VCP does not appear to be a common cause of familial or sporadic PDB in the absence of myopathy and dementia.

Keywords: Paget's disease; Ubiquitin; VCP genetic; NF-κB

Introduction

Paget's disease of bone (PDB [MIM 167250, 602080]) is a common disorder, affecting 3% of individuals over 55 years of age in the UK [23]. The cause of PDB is incompletely understood, but genetic factors play an important role, reflected by the fact that 15–40% of patients have at least one affected first-degree relative [18,20,21]. Several susceptibility loci for PDB have been identified by genome wide-search [9,10,15], and mutations in the Sequestosome 1 gene (*SQSTM1*) [MIM 601530] have emerged as a common cause of PDB in many populations [7,8,11–13,16].

Rarely, PDB can occur as part of an extended clinical syndrome, comprising of inclusion body myopathy and frontotemporal dementia (inclusion body myopathy, Paget's disease and frontotemporal dementia [14], IBMPFD; MIM 605382). The IBMPFD syndrome is inherited in an

* Corresponding author. Fax: +44 131 537 1051.
E-mail address: stuart.ralston@ed.ac.uk (S.H. Ralston).

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autosomal-dominant manner and was assigned to a 5.5 Mb region on chromosome 9p13 (maximum LOD score 3.64) by genome-wide scan in 2001 [14]. Subsequent positional cloning studies identified mutations in the Valosin-containing Protein (VCP) gene as the cause of IBMPFD [24]. VCP is widely expressed and is a member of the AAA-ATPase superfamily of proteins [25]. It has been implicated in the regulation of many cellular functions and is required for the proteasomal degradation of phosphorylated IκB-α [4,25], which is an essential step in NF-κB activation. Interestingly, the causative mutations in VCP all affect the highly conserved CDC48 domain, which is known to be involved in ubiquitin (UB)-binding [5,19]. This is particularly relevant since PDB-causing mutations in the *SQSTM1* gene also affect the ubiquitin (UB)-binding domain of the gene product, p62, suggesting that the disease processes in PDB and IBMPFD may be related. In this study, we evaluated the possibility that mutations or polymorphisms in VCP could cause or contribute to risk of typical PDB by analysis of the VCP gene in familial and sporadic cases of PDB.

Materials and methods

Patients

Patients with familial Paget's disease were recruited predominantly from clinic referrals in the UK, Australia and New Zealand as previously described [12]. Subjects with familial PDB included in this study were a subset of families previously described by Hocking et al. [12] in whom mutations in *SQSTM1* had been excluded by DNA sequencing. Subjects with sporadic PDB were recruited from routine clinic referrals in Aberdeen and Liverpool. In all cases, PDB was diagnosed on the basis of standard clinical criteria. Pedigree diagrams for all of the families included in this study are available on our website (http://www.abdn.ac.uk/medicine–therapeutics/bone/paget%20pedigrees.shtml). All subjects gave informed consent to being included in this study which was approved by the local research ethics committee.

Mutation and single nucleotide polymorphism screening

Genomic DNA was extracted from venous blood as described [12]. Mutation screening of coding exons and intron–exon boundaries of VCP was carried out using PCR primers and reaction conditions as previously described [24]. In three families, we conducted mutation screening of the whole gene, whereas in the remaining 41 families we focussed on exons 3, 5 and 6, which encode regions of the CDC48, Linker1 and AAA-ATPase domains, that have been reported to contain IBMPFD-associated mutations [24]. Mutation screening and genotyping were carried out by DNA sequencing on a MegaBace 1000 DNA sequencer (Amersham), using DYEnamic ET terminator chemistry according to the manufacturer's instructions. The primer sequences and annealing temperatures used for PCR amplification of all exons are shown in Table 1.

Association analysis

For the association study, we selected informative SNPs from within the VCP gene by interrogating the HapMap database [1]. We studied the patterns of linkage disequilibrium (LD) across the VCP gene using data from 90 Caucasians held in HapMap (Release #7, May 2004) and used the HAPLOVIEW program [2] to select tag SNPs that accounted for >90% of alleles at the VCP locus. Genotyping of tag SNPs in sporadic PDB cases and unaffected controls

Table 1
Primers for mutation screening of VCP exons

Region amplified	Primer (forward/reverse)	Tm
Exon 1	CTCGCCCCCTAGCTTCCCTTCCCTCTTT/GATTCGGCTCTTCTCGGCTCAGTCTCAG	64
	GTGGGCGAGCAGCGGCGACAAACC/GATAAACTCCGGAGCCAATGAACTG	59
Exon 2	TGCAAGAAAAATGAGAAAAGAAACCT/GTAGATACTGCTCCTCGTGACCT	55
Exon 3	GGCAGAACCAAAACCTAAAGACAAC/GAAGCCATCAATGAGGACAACAGT	55
Exon 4	CAGAATTAGCTCTCACCTTTCCG/ATGTGTGGCTCTTGATTTTGGC	55
Exon 5	GTTACCACATGATGCCACACTGA/CTAATGAAGGGCACTATCTAATGAGC	55
Exon 6	CAGGATTAGACATTGGGACAGG/GAATCTTCTTATACGGTAGGTTTTTG	54
Exon 7	ATGGGTGCAAAAAGGATGTGTTC/AGTTTCTCTGCCCTTAGTCTCCT	55
Exon 8–9	AAGGGCTTCAAGAGGATTAGGTG/AGGATGTGAGAAGTAGGCAGAGGTTA	55
Exon 10	AGATTACTTCCTTTCCCCTGTCCA/TTTTCCTTTACCCTACGTCCTGTCTA	55
Exon 11–12	AGTGCCTCAAACCTAAGAACAGTA/GGTCTTTGAGGCAGCATAGTAAGTAG	55
Exon 13	GTTGAGCAGCCAGCACTAAGAAT/ATGAAATAATGGAGGGGATGCT	55
Exon 14	ATCTTGTCCAGAAACTAAAGAGCACTC/CCAAAGTGCTGGGAATACAGGTG	57
Exon 15	CTCTTCTCGGCCTTATTCCAAAT/CAAGATTCCACTGGGGTTAGGGTT	55
Exon 16	GTACAAGAGCAAAGCCAAAAAAGAG/GGAAGGGCAAGGAGACCAATAAACT	55
Exon 17	ATGTTGATCAGGAGAGGAAGAAGGTG/AAGTTGGTTGGGAGCATTAGACAGTG	60
	TGCCCCAAACTACAACAGAAATG/GCGCCCACAGCCTGCTCCATTCTC	n/a

Primer sequences are written 5′ to 3′; Tm: annealing temperature in degrees centigrade.

was carried out by automated DNA sequencing, and the haplotypes were constructed from the genotype data in cases and controls using the PHASE haplotype analysis program (version 2.0.2) [22].

Statistical analysis

Linkage analysis at the VCP locus was carried out on genotype data generated by the ABI Prism Linkage Mapping Set MD-10. Families with a positive lodscore at markers flanking VCP were further analysed to identify haplotypes in the critical region that were shared by affected members of the family. The likelihood of linkage to the VCP locus was calculated by heterogeneity testing using HOMOG. Comparison of haplotype and genotype frequencies between cases and controls in the association study was done by χ^2 test. Power calculations indicated that the association study had greater than 80% power to detect a difference in allele frequency of 15% or more for the common haplotypes between cases and controls at a significance level of $P = 0.05$.

Results

Mutation screening of VCP in familial PDB

On linkage analysis, we identified 3 families out of the 44 available where there was a positive lodscore at the VCP locus and where there was haplotype sharing in affected subjects (Fig. 1). Although the P392L mutation in the *SQSTM1* gene had been found in two affected subjects from one of these families (FAM#51), two other affected individuals in this family did not carry an *SQSTM1* mutation, raising the possibility that both genes could have been involved in this family. The maximum lodscores at the nearest marker to VCP in these families were 0.83 (FAM#09), 0.83 (FAM#51) and 0.56 (FAM#61). We performed mutation screening analysis of all 17 exons in VCP in an affected member of each of these families, but no mutations were observed. We also performed a more limited mutations screen of exons 3, 5 and 6 of VCP for known IBMPFD-causing mutations in the remaining 41 families with PDB, but, again, no mutations were found, thereby excluding VCP mutations as a cause of familial PDB in our population.

Association study of VCP alleles in sporadic PDB

To investigate the possibility that polymorphisms at the VCP locus could contribute to risk of sporadic PDB, we analysed common haplotypes at the VCP locus in a series of 179 sporadic PDB patients and 172 age- and sex-matched controls. For this analysis, we selected 10 SNPs from the HapMap database and used the genotype data available for 90 individuals of European ancestry from the US to estimate the phase and frequency of common haplotypes at the VCP locus. We found that ~94% of alleles in this ethnic group were accounted for by three common haplotypes (Fig. 2). After examining patterns of LD across the gene, we found that 7 SNPs could be removed from the analysis on the basis of redundancy, thereby identifying three tag SNPs that described the most common haplotypes of VCP and which together accounted for ~94% of alleles at the locus. We

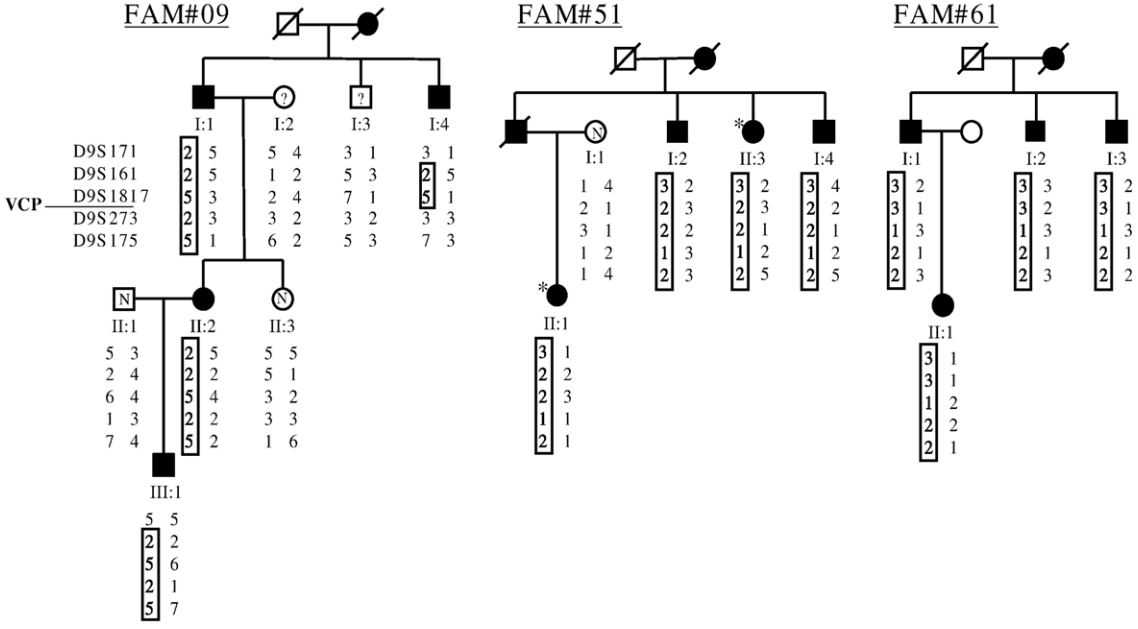

Fig. 1. Pedigree structures of families with positive lodscores and strong likelihood of linkage at the VCP locus on chromosome 9p13. The haplotype shown spans ~27 cM of chromosome 9p, and the VCP locus lies between markers D9S1817 and D9S273. The chromosomal segments shared by affected individuals are boxed. Individuals marked with an asterisk in FAM#51 carry P392L in the *SQSTM1* gene.

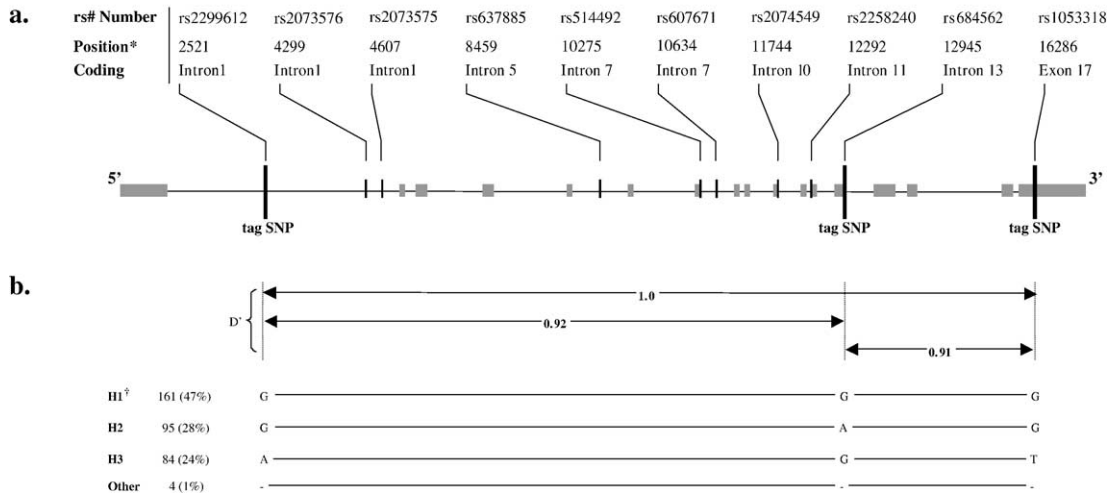

Fig. 2. HapMap SNPs and common haplotypes across the VCP locus. (a) The refSNP ID numbers of HapMap SNPs and positions within the VCP gene are shown. All of the selected SNPs are synonymous. Tag SNPs that can account for a large percentage of alleles at the VCP locus are indicated. (b) Linkage disequilibrium between the three tag SNPs are shown as D′ values. All three SNPs were in strong disequilibrium. Three common haplotypes with a combined frequency of >98% predicted from the tag SNPs are shown. Three other haplotypes (not shown) had a combined frequency of ~0.01. *SNP position relative to the start of Exon 1. †Numbers and frequencies of haplotypes estimated from 172 control individuals without PDB.

genotyped these tag-SNPs in 179 sporadic cases of PDB and 172 unaffected controls and found that they described three common haplotypes that accounted for >98% of alleles at the locus. We found no significant difference in the frequencies of these haplotypes among cases compared to controls ($\chi^2 = 1.1$; $df = 2$; $P = 0.576$; Table 2). We also analysed the data according to whether affected individuals or controls carried 0, 1 or 2 copies of each haplotype, but this similarly showed no association (data not shown).

Discussion

Mutations in the VCP gene have recently been shown to cause the syndrome of inclusion body myopathy, Paget's disease and frontotemporal dementia [24]. The role of VCP in the pathogenesis of PDB unassociated with myopathy and dementia is unknown, however. In this study, we conducted a full mutation screen of the VCP gene in three families where linkage analysis showed evidence of allele sharing at the VCP locus and a more limited mutation screen of exons 3, 5 and 6 in a further 41 families with familial PDB. No mutations were detected by this analysis, which indicates that in our population VCP does not contribute to the pathogenesis of familial PDB. We also wanted to determine if more subtle allelic variation in VCP might contribute to the pathogenesis of sporadic PDB. To investigate this possibility, we genotyped three tag SNPs that identified all common haplotypes at the VCP locus and studied allelic distribution in 179 patients with sporadic PDB and 172 age- and sex-matched controls. This study similarly failed to support the involvement of VCP in sporadic PDB since there was no difference in allele distribution for any haplotype between cases and controls. From this, it would appear that allelic variation in VCP is not an important cause of sporadic PDB.

Table 2
Haplotype frequencies in sporadic PDB cases and normal controls

N	179	172
Haplotype	Sporadic PDB	Controls
H1	180 (50.3%)	161 (46.8%)
H2	89 (24.9%)	95 (27.6%)
H3	82 (22.9%)	84 (24.4%)
Other	7 (1.9%)	4 (1.2%)

Haplotype frequencies are shown as the number and percent of the total alleles. There was no significant difference in allele frequency between the groups ($\chi^2 = 1.81$; $df = 3$; $P = 0.661$). The phases of the common haplotypes are shown in Fig. 1.

The IBMPFD-causing mutations in VCP localise within or close to the protein's ubiquitin-binding region, raising the possibility that similar mechanisms may account for the bone phenotype in VCP-mediated PDB as in *SQSTM1*-mediated PDB, where the causative mutations also cluster in the ubiquitin-associated domain [7,8,11–13,16]. Whilst the mechanisms by which these mutations cause PDB remain to be fully elucidated, functional studies have shown that PDB-causing mutations in *SQSTM1* result in loss of function with regard to ubiquitin binding, but only in the context of the whole *SQSTM1* protein [3].

There is no information on the functional effects of the VCP mutations that cause IBMPFD, but we previously reported that VCP is present within the inclusion bodies in muscle cells from these patients [24]. It is currently unknown whether VCP is also contained within the neuronal inclusion bodies in IBMPFD, but many neurodegenerative conditions are associated with inclusion bodies that contain ubiquitin, presumably as the result of abnormalities in ubiquitin-mediated protein degradation [17]. In view of this, we speculate that the multi-organ involvement that occurs in patients with IBMPFD signals the importance of VCP as a

mediator of protein degradation in several organ systems, whereas the effect of *SQSTM1* mutations seems to be more restricted to cells of the osteoclast lineage. In keeping with this, mice with targeted deletion of *SQSTM1* have no obvious phenotype, except when challenged by bone-resorbing factors like PTHrP when they show defective osteoclastogenesis compared to wild type mice [6]. Whilst the bone phenotype observed in our PDB patients is similar to that observed in IBMPFD, none of our sporadic patients had been diagnosed as suffering from myopathy and dementia nor was there a history of this in the families we studied. Although this study shows that VCP is not an important cause of PDB in the absence of dementia and myopathy, identification of VCP as the causal gene for IBMPFD emphasises the importance of the ubiquitin–proteasome pathway in bone cell function in general and in PDB in particular.

Electronic database information

URL's for databases used in this article are as follows:

Online Mendelian Inheritance in Man (OMIM) for PDB [MIM 167250, 602080] http://www.ncbi.nlm.nih.gov/OMIM.

National Centre for Biotechnology Information (NCBI) for reference sequences and refSNP information http://www.ncbi.nlm.nih.gov/SNP.

University of Aberdeen (for pedigree diagrams) http://www.abdn.ac.uk/medicine–therapeutics/bone/paget%20pedigrees.hti.

International Haplotype Mapping Consortium for genotype data for VCP SNPs http://www.hapmap.org/.

Prof. Matthew Stephens, University of Washington for PHASE haplotype analysis software http://www.stat.washington.edu/stephens/software.html.

JC Barrett and colleagues, The Broad Institute, MIT, for HAPLOVIEW haplotype analysis software http://www.broad.mit.edu/mpg/haploview.

Prof. Jurg Ott, Rockefeller University for HOMOG heterogeneity analysis program http://www.linkage.rockefeller.edu/.

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